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A composition used as a therapeutic agent against chronic viral hepatic diseases.

A combination, comprising at least one polypeptide sequence, mediating the antigenicity of one or more epitopes, and a carrier, capable of presenting this/these polypeptide sequence(s), which are useful for the production of a medicament for the treatment of chronic viral hepatitis, is provided.

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The present invention relates to a composition comprising a polypeptide sequence prepared by recombinant DNA techniques and a carrier to provide a curing agent against chronic viral hepatic diseases. The invention relates to DNA sequences coding for said polypeptide sequences and to transfected cells for the expression of the same.

At least five different viruses, namely Hepatitis virus A, B, C, D and E, are able to trigger the clinical aspect of an acute hepatitis. Hepatitis A and E, which are transferred enterically, always heal, whereas hepatitis B, C (formerly called parenteral hepatitis Non-A Non-B), and D can progress into a chronic stage of inflammation, which in turn can result in liver cirrhosis and primary hepatocellular carcinoma.

There is relatively little data available on hepatitis C and D, on methods for the diagnosis and their treatment and on the respective viruses. The hepatitis D virus is a RNA virus which is known to be incomplete. Therefore, it needs a helper virus to develop in patients and is found only in individuals infected with HBV. Only very recently the hepatitis C virus has been detected, and an antibody test (anti-HCV) facilitating the diagnosis of chronic hepatitis C infections has been developed. However, there is an increasingly urgent need for a treatment to cure this disease.

The same holds true for chronic hepatitis B, a much better studied disease with respect to its recognition by immunological methods, its causative virus and the viral life cycle and DNA sequence. Patients are said to be chronic carriers of the hepatitis B virus if the viral DNA persists longer than ten weeks, the HBe-antigen (HBeAg) for more than 12 weeks, or if the hepatitis B surface-antigen (HBsAg) is persistent longer than six months.

Roughly three hundred million people are deemed to suffer from chronic hepatitis B, most of them living in the Far East.

For these people the main risk to be infected appears to be during or immediately after birth, since a chronically infected mother transfers the virus to her newborn. 90 percent of the children infected this way will become chronically infected, too, during later life. In the Western World infection occurs more commonly later in life, during childhood or even adulthood, mainly by a parenteral or sexual transmission. In these cases of hepatitis B infection after birth only five to ten percent of the infected become chronic carriers. The virus transferred, however, is not responsible for distinct reactions shown by infected people either to eliminate the virus or to retain it in the body lifelong. Consequently, it seems to be a matter of the immunological status that determines the future physical condition.

The HB-virion (Dane particle) is composed of different structural proteins, the core proteins and the surface (S) proteins. The latter are translation products of an open reading frame encompassing the coding sequence of three S-type domains, each of which starts with an ATG triplet capable of initiating translation in vivo. The domains are referred to as preS1, preS2 and S in the order of 5' to the 3' end of the molecule. There are six protein products derived from this ORF: a glycosylated and a non-glycosylated form of the major protein (gp27 and p24) translated from the S domain only (226 amino acids), a middle protein (281 amino acids) having one or two polysaccharide side chains (gp33 and gp36, respectively), that is encoded by the preS2- and S-region, and finally, both a glycosylated (gp42) and a non-glycosylated (p39) form of the large protein (389-400 amino acids, depending upon the viral serotype), which is formed by translation of preS1, preS2 and S. The core proteins are HBcAg and HBeAg, the latter one conceivably being a processing product of HBcAg.

The Dane particle, which is the infectious virion, comprises both core and surface proteins, whereas the filaments consist of a mixture of the six surface antigens. The S peptides alone assemble to form the so-called 20nm particles, which are completely uninfecious.

Patients infected by the HB virus pass through several stages of the hepatitis, before they are regarded to be chronically HBV-infected. Immediately after infection an infectious stage will follow, characterized by the presence of HBeAg in the serum. Continued HBs antigenaemia in spite of inhibited HBV replication indicates the presence of viral DNA sequences integrated into the cellular genome of the patient. The integrated viral sequences do not enable the host cell to synthesize the complete virus. However, liver cells having HBV-sequences integrated are capable of producing HBsAg only, which in turn is detectable in the serum of the patient and is an indicator for chronic hepatitis B. Most probably the transformed hepatocytes are not lysed by cytotoxic T-cells, but proliferate and induce either chronic persistent hepatitis (CPH) or chronic active hepatitis (CAH), which may then proceed to cirrhosis of the liver or to primary hepatocellular carcinoma resulting in premature death of the patient.

Recently it has been established that patients who are chronically HBV infected show a defect in endogenous interferon production (Abb et al., 1985: J. Med. Virol 16, 171-176). This was the rationale to treat patients suffering from chronic hepatitis B, as indicated by the presence of HBeAg and HBV-DNA in the serum, with interferon α (IFN α). Controlled trials with large numbers of patients showed that the administration of interferon α resulted in significantly increased elimination of the hepatitis B-virus, when

compared to controls. However, persons infected at or around time of birth do not appear to seroconvert in response to this therapy. This phenomenon unfortunately precludes some 75% of carriers from IFN α therapy.

At present, the exact mode of action of interferon α on chronic hepatitis B remains unclear. Its antiviral activity might protect infected cells from infection or reduce viral transcription, translation and replication in HBV-infected cells. Interferon further has immunomodulatory effects by activating T-cells, macrophages and NK-cells and by inducing the expression of MHC class I proteins.

Another approach to treat chronic hepatitis B is based on the idea to inhibit replication of the virus, thus impairing its defence sufficiently to render the host immune system capable of eliminating the virus. This led to test antiviral drugs such as adenine arabinoside and adenine arabinoside monophosphate for treatment of chronically HBV infected individuals. However, less than half of the patients responded to this therapy, either by sustained or transient seroconversion (HBeAg $^{+}$ to anti-HBe $^{+}$). A further negative aspect of these antiviral drugs are their immunosuppressive properties. Other drugs that have been tested for treatment of chronic carriers include interferon β and γ , acycloguanosine (acyclovir), interleukin 2, steroids, such as prednisolone, and combinations thereof. But none of them could provide better results than treatment with interferon α . Only a combination therapy, including the initial administration of steroids followed by that of IFN α may increase the response rate in selected patients.

It is known from the prior art, that chronically HBV infected chimpanzees can not be cured by treatment with HBsAg (bound to a tetanus toxoid) nor with anti-HBs antibodies. Furthermore, it has been attempted to immunize chronically HBV infected patients by administration of S peptides. This treatment did not even result in anti-HBs antibody formation in these persons.

Additionally, according to the definition, chronic carriers of hepatitis B virus are characterized in that HBsAg is detectable in their serum. Therefore, it has been absolutely unforeseeable, that a combination, comprising a T-cell activating epitope of the viral S peptide, according to the present invention, is able to induce an immunization in and a final healing of chronic carriers of hepatitis virus B.

Considering the above-discussed state of the art it is the objective of the present invention to provide an effective therapeutic agent for the treatment of viral chronic hepatic diseases which leads to a complete response (i.e. to the sustained inhibition of HBV-replication, the loss of HBV DNA and DNA polymerase and to a decrease and finally the disappearance of HBeAg and HBsAg in the serum of patients).

According to the present invention this goal is achieved by a combination of a) at least one polypeptide sequence mediating the antigenicity of one or more epitopes and b) a carrier, capable of presenting the epitope sequence(s) a), wherein the polypeptide sequence(s) a) can be bound to carrier b) by adsorption, any chemical bonding or secondary valences.

This invention is furthermore directed to the use of this combination for the production of a medicament for the treatment of chronic viral hepatitis.

It is important that polypeptide sequence a), which may be one or more different polypeptides, mediates the antigenicity of a T cell-activating epitope in a direct or indirect way. According to the present invention polypeptide sequence(s) a) may be a polypeptide or a combination of two or more polypeptides of hepatitis B virus of any subtype, particularly adw, ayw, adr and ady.

These peptides derived from hepatitis B virus may be HBV peptides preS1, preS2 or S or the HBV core antigens.

Useful as polypeptide sequence(s) a) are furthermore any of the above-stated polypeptides or a combination of two or more polypeptides which are modified either by amino acid deletions, whereby at least one epitope comprising at least six consecutive amino acid residues must be preserved, or by adding further amino acids either at the N-terminus, the C-terminus or as insertions into the polypeptide sequence(s) a). In each of these cases it is essential, however, that the biological activity is maintained.

Preferably, polypeptide sequence(s) a) is myristylated.

In order to display the appropriate pharmacological activity it is necessary that in the combination of the present invention polypeptide sequence(s) a) is presented on a carrier b). This carrier consists of a particular substance which for example may consist of particles of a hydrophobic polymer, of inorganic particles, or of particles of a polysaccharide. Preferably, carrier b) is a second polypeptide sequence which forms particles upon secretion, said particles having preferably a diameter of at least 10nm.

It is preferred that the particle forming polypeptide sequence b) is a substantial part of or the complete amino acid sequence of a polypeptide which may be selected from HBV S peptide, HBV core antigen, HAV core antigen, HAV surface antigen, HIV surface antigen and HIV core antigen as well as the surface antigen of polio virus. Preferred as the particle-forming carrier b) is HBV S peptide and/or core peptide.

When used as the carrier sequence b) the above-stated polypeptides may be modified by arbitrary deletions of amino acids, by substitutions of one or more amino acids or by adding one or more amino

acids either at the N-terminus, the C-terminus or by insertion of one or more amino acids into the polypeptide sequence b), provided that the particle-forming capacity is maintained. Preferably, polypeptide sequence b) is myristylated.

If the carrier b) is a polypeptide sequence, both sequences a) and b) may be linked via one or more of the following interactions: hydrophobic anchoring (mediated by myristic acid), disulfide bridge formation, or both sequences may be connected by a peptide bond to form a fusion peptide. In the latter case optionally a spacer sequence may be inserted between polypeptide sequence(s) a) and polypeptide sequence b), which spacer sequence is linked to both polypeptides via peptide bonds.

The present invention furthermore provides a recombinant DNA molecule coding for a combination, that is useful for production of a medicament to treat chronic viral hepatic diseases. The recombinant DNA molecule comprises at least one first DNA sequence, optionally a second, a third and/or a fourth DNA sequence wherein

- i) said at least one first DNA sequence codes for at least one polypeptide sequence a) as defined above,
- ii) said second DNA sequence codes for a polypeptide sequence b) according to the above definition of the particle forming peptide,
- iii) said third DNA sequence codes for a spacer sequence, and
- iv) said fourth DNA sequence codes for a selection marker,

and wherein the DNA sequences are controlled by DNA elements essential for expression, and optionally have a common reading frame.

On account of the fact, that many amino acids are designated by more than one triplet, there exist several DNA sequences embraced by the present invention, which code for the above-defined peptide sequences a) and b). Apart from this, the invention further embraces recombinant DNA molecules, which differ from the above-defined recombinant DNA molecules by the fact, that up to 30% of the nucleotides may be substituted.

A further object of the present application is to provide a host cell transfected with a recombinant DNA molecule coding for the above combination, which is useful for treatment of chronically HBV-infected patients. This host cell may be a mammalian, a yeast or a bacterial cell. For the purpose of this invention it is preferred, that this cell does not produce any human serum proteins or any primate serum proteins other than the polypeptide(s) being comprised within the above combination.

The term "HBV S peptide" as used herein refers to the peptide encoded by the entire region of the HBV genome. The term "HBV pre-S2 peptide" as used herein refers to the peptide encoded by the entire pre-S2 and S regions of the HBV genome. The term "HBV pre-S1 peptide" as used herein refers to the polypeptide encoded by the entire pre-S1, pre-S2 and S regions of the HBV genome. The term "epitope" as used herein refers to a sequence of at least six consecutive amino acids encoded by the designated genome region (e.g. a "HBV pre-S2 epitope" refers to a sequence of at least six amino acids encoded by the pre-S2 region of the HBV genome). The term "T-cell epitope" as used herein refers to an epitope that interacts with receptors on the surface of T-cells to enhance or otherwise effect an immune response.

As used herein "antigenicity" means the ability to provoke an immune response (e.g. acting as an antigen), the ability to cause the production of antibodies (e.g. acting as an antigen) and/or the ability to interact with a cell surface receptor so as to enhance an immune response or production of antibodies.

The term "HBV" means any subtype of the virus, particularly adw, ayw, adr and ayr, described in the literature (P. Valenzuela, Nature Vol. 280, p. 815 (1979), Gerlich, EP-A-85 111 361, Neurath, EP-A-85 102 250). Examples of peptide sequences thereof, constituting polypeptide sequence(s) a), which mediate the antigenicity of one or more epitopes, are shown in the Sequence Listing (SEQ ID No. 17-20, 22).

Preferred embodiments of the present invention are the following combinations:

- HB S-antigen particles with specific epitopes (determinants) of the pre-S1-, pre-S2-, and/or core peptides;
- HB core-antigen particles with specific epitopes (determinants) of the pre-S1-, pre-S2-, S-peptide, and/or of the core antigens;
- Hepatitis A-antigen particles with specific epitopes (determinants) of the hepatitis B S-, pre-S1-, pre-S2-, and/or core-peptides.

Recombinant DNA molecules preferred for the present invention are characterized by the presence of sequences coding for polypeptide sequence(s) a), mediating the antigenicity of one or more T-cell epitopes, and for polypeptide b), which upon secretion forms particles having a diameter of 10nm or more, both of which are under control of a suitable promoter. As examples for sequences coding for a) there may be mentioned any of the sequences listed under ID numbers 1 to 24 in the sequence Listing. Examples for the DNA sequence coding for polypeptide sequence b) are represented by any of the ID-sequences 25 to 27 in the Sequence Listing.

Any of the 24 sequences (ID numbers 1 to 24) may be combined to any sequence disclosed under ID number 25 to 27 in the Sequence Listing, therein both orders a-b and b-a are included.

Hepatitis virus sequences used in the recombinant DNA construct of the present invention can be formed or isolated by any means including isolation and ligation of restriction fragments, chemical synthesis of oligonucleotides using a synthesizer (Cyclon, Bio-Search), and synthesis by the PCR method (T.J. White, N. Arnleim, H. E. Erlich, 1989; The Polymerase Chain Reaction, Technical Focus 5 (6)).

Preferred recombinant DNA molecules were formed by the ligation of synthetic oligonucleotides to a 5' XbaI-BglII 3' fragment (ID number 27) from the S region of the HBV genome, which is derived from a BglII-BglII HBV fragment including the entire pre-S1-pre-S2-S-region, or to the entire S-region. Oligonucleotides used in making such constructs are summarized in Table I below.

Table I

Function	Definition	SEQ ID No.
core (adw)	aa 59-87	6
core (adw)	aa 2-28	7
core (adw)	aa -10-28	8
core (adw)	aa 29-58	9
core (adw)	aa 1-87	10
core (adw)	aa -10-87	11
core (adw)	aa 70-110	12
core (adw)	aa 80-125	13
core (adw)	aa 88-120	15
S1 (ayw)	aa 9-28	17
S1 (ayw)	aa 83-103	18
S1 (ayw)	aa 20-40	19
S1 (ayw)	aa 59-94	20
S1 (adw)	aa 94-114	21
S1 (adw)	aa 70-105	22
S2 (ayw)	aa 2-21	23
S2 (ayw)	aa 14-33	24

* aa = amino acid

Other preferred DNA molecules were formed by ligation of core sequences, which are prepared by the PCR method and which code for T-cell epitopes, to the core sequence of HBV (SEQ ID NO 25) functioning as polypeptide sequence b). Oligonucleotides used in preparing these constructs are given in Table II-1.

Table II-1

Function	Definition	SEQ ID No.
core	complete, bp 1901-2500	1
core	C-terminal deletion, bp 1901-2405	2
core	C-terminal deletion and stop codon inserted, bp 1901-2405	3
core:precore	10 aa precore, C-terminal deletion, bp 1871-2405	4
core:precore	10 aa precore, C-terminal deletion and stop codon inserted, bp 1871-2405	5
core	aa (-10-120)	16
core:precore	10 aa precore, complete core, bp 1871-2500	35

Table II-2 shows several examples, where the T-cell epitope-coding DNA sequences have been isolated by restriction fragmentation of the HBV genome and have been ligated to the DNA sequence coding for polypeptide sequence b) as defined above.

Table II-2

Function	Definition	SEQ ID No.
core/precore	complete, bp 1403-31	--
S2 ay/ad		--
S2 (K) ay/ad	S2-S, 7 codons deleted, start codon ATG changed to ATA	14

-- Sequence has been published by Galibert, F. et al. (1979: Nature 281, 646-650) and by Ono, Y. et al. (1983: Nucl. Acid Res. 11(6), 1747-1757)

In Table II-3 specific recombinant DNA molecules are listed. The procedure for their construction will be described in more detail in the Examples.

Table II-3

Final construct	T-cell epitope	Particle Former	Selection Gene
MT-core(-10-120) + SAg + neo	core(aa-10-120)	S adw/ayw or S/XbaI/BglII	neo
MT-S1(aa 9-28)-S + egpt	S1(aa 9-28)ay	S adw/ayw or S/XbaI/BglII	egpt#
MT-core-neo	core/precore bp 1403-31	core adw	neo
MT-core(1-87) + HBsAg - neo	core(aa 1-87)	S adw/ayw or S/XbaI/BglII	neo

egpt = E coli xanthine guanine phosphoribosyl transferase

Preferred recombinant DNA molecules according to the present invention comprise, apart from the regions coding for polypeptides a) and b), an additional DNA sequence coding for a selection marker. Furthermore, they comprise all usual elements essential for the expression, such as promoter sequence, start codon and a polyadenylation signal.

Examples of suitable promoters are the methallothionein (MT), the U2 and the H2K promoter in case of using mammalian cells as a host cell. If yeast or bacterial cells are to be employed, appropriate yeast and bacterial promoters, such as the GCN4- and the GAL 1/10 promoter or the prokaryotic trp- and tac promoters, respectively, may be used.

In order to produce the combination of polypeptide(s) a) and polypeptide b) according to this application the recombinant DNA molecule is inserted into host cells by transfection (in case of mammalian cells), by transformation (in case of yeast and bacterial cells), or by other means. As a host cells of any organism may be used that are capable of transcribing and translating recombinant DNA molecules, such as mammalian, bacterial and yeast cells.

Suitable mammalian cells according to this invention are for example VERO cells (a monkey kidney cell line), 3T3-, C127 and L cells (murine fibroblast cell lines), and CHO (Chinese hamster ovary) cells, which are either positive or negative in dehydrofolate reductase.

According to a specific embodiment of the present invention it is furthermore possible that the above-defined first DNA sequence and the above-defined second DNA sequence, which code for polypeptide sequence(s) a) and for a polypeptide sequence b), respectively, are present in different recombinant DNA molecules, in which case the host cells are cotransfected with both of these recombinant DNA molecules.

Tab. III Possible alternatives of compositions for particles containing T cell epitopes for targeting chronic hepatitis carrier

	FINAL CONSTRUCT	PROMOTER	SYN	T-CELL-EPIOTOPE		GENE	PARTICLE FORMER	SELECTION GENE	PURIFICATION
				PCR					
12		MTA12A2	Core (MA 10 - 87)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
13		MTA12A2	Core (MA 70 - 110)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
14		MTA12A2	Core (MA 80 - 125)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
15		MTA12A2	Core (MA 88 - 120)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
16		MTA12A2	Core (MA 10 + SA16 + MA 2 - 87)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
17	MT - core (-10 - +120) + SA9 + neo	MTA12A2			Core (MA 10 - +120)		entire S adw/zyw S/XbaI/RgIII	neo/egpt	
18	MT - S1 (MA 9-28) - S + egpt	MTA12A2	S1 - (MA 9 - 28) (zy)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
19		MTA12A2	S1 - (MA 83 - 103) (zy)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
20		MTA12A2	S1 - (MA 20 - 40) (zy)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	Materials and Methods
21		MTA12A2	S1 - (MA 59 - 94) (zy)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
22		MTA12A2	S1 - (MA 94 - 114) (ad)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
23		MTA12A2	S1 - (MA 70 - 105) (ad)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
24		MTA12A2	S1 - (MA 9 - 28) (zy)				core adw	neo/egpt	
25		MTA12A2	S2 - (MA 2 - 21) (zy)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
26		MTA12A2	S2 - (MA 14 - 33) (zy)				entire S adw/zyw S/XbaI/RgIII	neo/egpt	
27		MTA12A2				S2 zyw	S zyw/adw	neo/egpt	
28		MTA12A2	Adapter			S2 - (K) - zyw	S zyw/adw	neo/egpt	

Tab. III Possible alternatives of compositions for particles containing T-cell epitopes for targeting chronic hepatitis carrier

1	2	T-CELL EPITOPE			PARTICLE FORMER	SELECTION GENE	PURIFICATION
		SYN	PCR	GENE			
1	PROMOTER						
	MI/12/202		Core without pre core e.g. bp 1901 - 2500		Core (adv)	neo/egpt	
2	MI/12/202		Core without pre core; with deletion of the C-terminus e.g. bp 1901 - 2405		Core (adv)	neo/egpt	
3	MI/12/202		Core without pre core; with deletion at the C- terminus + stop signal e.g. bp 1901 - 2405		Core (adv)	neo/egpt	
4	MI/12/202		Core and pre core 10 AA e.g. bp 1871 - 2405	Core with pre core 1e bp 1403 - 31	Core (adv)	neo/egpt	Materials and Methods
5	MI/12/202		Core and pre core 10 AA; with deletion at the C-terminus e.g. bp 1871 - 2405		Core (adv)	neo/egpt	
6	MI/12/202		Core and pre core with deletion at the C- terminus + stop signal e.g. bp 1871 - 2405		Core (adv)	neo/egpt	
7	MI/12/202	Core (AA 59 - 87)			entire S adv/advw S/XbaI/BglII	neo/egpt	
8	MI/12/202	Core (AA 2 - 28)			entire S adv/advw S/XbaI/BglII	neo/egpt	
9	MI/12/202	Core (AA 10 - 420)			entire S adv/advw S/XbaI/BglII	neo/egpt	
10	MI/12/202	Core (AA 29 - 58)			entire S adv/advw S/XbaI/BglII	neo/egpt	
11	MI/12/202	Core (AA 1 - 87)			entire S adv/advw S/XbaI/BglII	neo/egpt	

Notes: 1 see example 3
2 any of the stated promoters is suitable
3 see examples

Table III gives an overview on how to combine suitable DNA sequences to get DNA constructs according to the present invention. It is to be noted that any constituents disclosed in this table may be combined to provide a DNA sequence which may be taken, if transfected into a host cell, to produce a combination (comprising polypeptides(s) a) and b)) as a medicament for the treatment of chronic viral hepatitis. The DNA sequences coding for the T-cell epitope sequences have been prepared synthetically (SYN) with a Bioscience Cyclon synthesizer, by PCR procedure (PCR), or by restriction enzyme fragmentation of the viral genome (GENE).

For the treatment of patients suffering from chronic viral hepatitis the combination of polypeptide

sequence(s) a) and a carrier b) may be formulated in any type of a pharmaceutical composition, which furthermore comprises a suitable diluent or pharmaceutical carrier material, such as a buffer solution.

The administration may be effected by any method, i.e. by parenteral (e.g. intravenous or intramuscular) or oral (e.g. by using typhoid bacterial cells to encapsulate the active substance) administration.

5 The pharmaceutical preparation comprises the above-described combination in sufficient concentration to elicit a response upon administration.

Brief Description of the Figures

- | | | |
|----|--------------------|--|
| 10 | Figure I | shows a DNA construct, coding for a promoter, a particle former sequence and a selection gene (described in Example 3/4). |
| | Figure II | shows a DNA-gene construct containing a promoter, an epitope with the entire HB-S-Ag and a selection gene (described in Example 3/18). |
| | Figure III | shows a DNA construct presenting a promoter, a T-cell epitope with a particle former |
| 15 | | residue and a selection gene (described in Example 3/21). |
| | Figure IV | shows the AST values of chimpanzee 1 during the Hepa-Care treatment (described in Example 10/1). |
| | Figure V | shows the antigen values of chimpanzee 1 during the Hepa-Care treatment (described in Example 10/1) |
| 20 | Figure VI | shows values of liver enzymes ALT and GGT of chimpanzee 1 booster treated three times with Hepa-Care (described in Example 10/2). |
| | Figure VII | shows values of liver enzymes ALT, AST, and GGT and of antigen of chimpanzee 2 during the Hepa-Care treatment (described in Example 10/3). |
| | Figure VIII | shows the liver enzymes as determined for an untreated control chimpanzee (described in Example 10/3). |
| 25 | Figures IX & X | show the antigen and antibody titers of patient 1 during the Hepa-Care treatment, respectively (described in Example 11). |
| | Figures XI & XII | show the antigen and antibody titers of patient 2 during the Hepa-Care treatment, respectively (described in Example 11). |
| 30 | Figures XIII & XIV | show the antigen and antibody titers of patient 2 during the Hepa-Care treatment, respectively (described in Example 11). |

The invention is more specifically described by the following examples.

Example 1

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1. Fractionated precipitation with polyethylene glycol (PEG)

The supernatant of HBV protein producing cultures was collected and split into portions of 2,400 ml. To each portion 144 g of PEG 6000 (Serva) were added and dissolved by stirring at room temperature for 20

40 minutes and was stirred for another 6 hours at 4° C. The precipitate was separated by centrifugation in 500 ml bottles in a GS 3 rotor at 9,000 rpm (15,000 x g) for 30 minutes at 10° C. The supernatant was collected and 144 g of PEG 6000 were added and dissolved as described above. The solution was stirred at 4° C for 3 hours. The precipitate from this solution was harvested as described above except that centrifugation was continued for 60 minutes.

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2. Gel Chromatography

The material obtained after PEG precipitation was redissolved in 20 ml PBS and submitted to gel chromatography on A-5m (BioRad). Column dimensions were 25 x 1000 mm and 480 ml bed volume. In a

50 typical fractionation run 1,000 µg of PEG precipitated HBV protein in 10 to 15 ml was loaded and eluted with PBS at a speed of 6 drops/min (18 ml/h). 3 ml fractions were collected. HBV protein eluted with the first peak. Collected fractions were submitted to a CsCl gradient.

3. Sedimentation in CsCl gradient

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About 30 fractions covering the first peak in column chromatography on A-5m and containing prepurified HBV protein were collected to approximately 100 ml. This solution was adjusted to a density of 1.30 g/cc with CsCl and subsequently transferred to a polyallomer tube fitting into a SW 27.28 rotor

(Beckman). A gradient was set by underlaying 4 ml of a CsCl solution of 1.35 g/cc and by overlaying 4 ml of 1.25 g/cc followed by 4 ml of 1.20 g/cc density. This gradient had been run at 28,000 rpm for 50 hours at 10° C. Thereafter the gradient was fractionated, and purified HBV protein floating in the 1.20 g/cc density layer was collected. The solution was desalted by three cycles of dialysis in bags against water.

Example 2

Quantitative Determination of HBV protein

1. with Radioimmunoassay

In the AUSRIA II-125 "sandwich" radioimmunoassay (commercially available from Abbot), beads coated with guinea pig antibody to Hepatitis B surface antigen (Anti-HBs) were incubated with serum or plasma or purified protein and appropriate controls. Any HBsAg present was bound to the solid phase antibody. After aspiration of the unbound material and washing of the bead, human 125T-Anti-HBs was allowed to react with the antibody-antigen complex on the bead. The beads were then washed to remove unbound ¹²⁵I-Anti-HBs.

)-Anti-HBs HBsAg

)-Anti-HBs . HBsAg ¹²⁵I-Anti-HBs

)-Anti-HBs . HBsAg . ¹²⁵I-Anti-HBs

The radioactivity remaining on the beads was counted in a gamma scintillation counter.

2. with ELISA

In the Enzygnost HBsAg micro "sandwich" assay (commercially available from Behring), wells were coated with anti-HBs. Serum plasma or purified protein and appropriate controls were added to the wells and incubated. After washing, peroxidase-labelled antibodies to HBsAg were reacted with the remaining antigenic determinants. The unbound enzyme-linked antibodies are removed by washing and the enzyme activity on the solid phase was determined. The enzymatically catalyzed reaction of hydrogen peroxide and chromogen was stopped by adding diluted sulfuric acid. The colour intensity was proportional to the HBsAg concentration of the sample and was obtained by photometric comparison of the colour intensity of the unknown samples with the colour intensities of the accompanying negative and positive control sera.

Example 3

Preparation of gene constructs of the present invention containing promoter, desired antigen sequences and selection gene.

1. Isolation of the MT-promoter.

The plasmid pBPV-342-12 (ATCC 37224) was digested with the endonucleases BglII and BamHI. Three DNA molecules were generated. The fragment of interest contains the methallothionein promoter and a pBR322 sequence comprising 4.5 kb and is easily detectable from the other fragments (2.0 kb and 7.6 kb).

The reaction was performed in a total volume of 200 µl of reaction buffer at a final concentration of 0.5 µg/µl DNA including 100 units of each restriction enzyme. The completion of the digestion was checked after incubation at 37° C for three hours by agarose gel electrophoresis at a 0.8% agarose gel. The reaction was stopped by adding 4 µl 0.5 M EDTA.

The 4.5 kb fragment was separated from the other fragments by preparative 1.2% agarose gel electrophoresis. The DNA was eluted from the agarose gel on DE-81 Whatman filter paper from which the DNA was removed in a high salt buffer. The DNA was purified by a phenol-chloroform extraction and two ethanol precipitations.

2. Ligation of a 1.8 kb fragment coding for the HBV-core-antigen.

A 1.8kb BamHI-BamHI fragment, containing the HBV-core coding regions was isolated from HBV-containing DNA. This fragment was ligated together with the 4.5 kb fragment containing the MT-promoter and the pBR residue (described in 1).

2 µl of the 1.8 kb fragment were mixed with 3 µl of the 4.5 kb fragment and ligated together in a total volume of 10 µl ligation buffer, containing 2 units T4-DNA ligase and 2 mM ATP at 14° C overnight.

The ligation mixture was added to 150 µl competent bacterial cell suspension for DNA up-take. After the DNA up-take the bacterial cells were spread on LB agar plates containing 50 µl/ml ampicillin at volumes of 50 to 300 µl cell suspension per plate. The agar plates were incubated at 37° C overnight. Single isolated bacterial colonies were screened for the presence of a plasmid containing the desired

fragments.

3. Screening for desired plasmid containing bacterial colonies.

Single colonies were picked with a toothpick and transferred to a LB-ampicillin medium containing tube (5 ml). The tubes were incubated overnight at 37° in a rapidly shaking environment. A mini-plasmid preparation of each grown bacterial suspension was made. The different resulting DNAs were proved by digestion with the restriction endonuclease BglII. Two molecules were expected, a 400 bp fragment and a 5.9 kb fragment. The digestion was analysed by agarose gel electrophoresis. Plasmid DNA was isolated from the bacterial cells.

4. Insertion of a neomycin selection marker.

The plasmid resulting from (3) above was linearized by digestion with the restriction enzyme EcoRI. The reaction was performed in a total volume of 50 µl and a final concentration of 1 µg/µl plasmid DNA. 50 units of EcoRI were added and the digestion was proved after incubation at 37° C for three hours by agarose gel electrophoresis. The reaction was stopped by adding 1 µl of 0.5 M EDTA and the DNA was precipitated with a final concentration of 0.3 M sodium acetate and 3-4 volumes of ethanol at -80° C for 30 minutes. The precipitated DNA was dissolved in 50 µl distilled water.

2 µl of the linearized plasmid was mixed with 3 µl of the DNA fragment containing the methallothionein promoter and the neomycin selection gene (isolated from the plasmid pMT-neo-E (available from ATCC/Exogene) by digestion with the endonuclease EcoRI as a 3.9 kb fragment), and ligated together. Single bacterial colonies were screened for the presence of the desired plasmid.

5. Isolation of a fragment containing the U2 promoter sequence.

The plasmid pUC-8-42 (available from Exogene) was digested with the restriction endonucleases EcoRI and Apal. Two DNA molecules were generated. The fragment of interest contains the U2-promoter comprising 340 bp and is easily detectable from the other fragment (3160 bp). The digestion was performed in a total volume of 200 µl reaction buffer at a final concentration of 0.5 µg/µl DNA including 100 units of each restriction enzyme. The completion of the digest was checked after incubation at 37° C for three hours by agarose gel electrophoresis in a 0.7% agarose gel. The reaction was stopped by adding 4 µl 0.5 M EDTA. The 340 bp fragment was separated from the plasmid DNA by preparative 1.2% agarose gel electrophoresis. The DNA was eluted from the agarose gel on DE-81 Whatman filter paper from which the DNA was removed in a high salt buffer. The DNA was purified by a phenol/chloroform extraction and two ethanol precipitations.

6. Insertion of the fragment containing the promoter sequence into a polylinker plasmid.

The plasmid pSP165 (commercially available from Promega Biotec) containing a polylinker sequence (containing the following restriction sites: EcoRI, SacI, SmaI, Aval, BamHI, BglII, Sall, PstI, HindIII) was linearized with the restriction enzyme EcoRI.

The reaction was performed in a total volume of 50 µl and a final concentration of 1 µg/µl plasmid DNA. 50 units of EcoRI was added and the digestion was proved after incubation at 37° C for three hours by agarose gel electrophoresis. The reaction was stopped by adding 1 µl of 0.5 M EDTA and the DNA was precipitated with a final concentration of 0.3 M sodium acetate and 3-4 volumes of ethanol at -80° C for 30 minutes. The precipitated DNA was dissolved in 50 µl distilled water.

2 µl of plasmid DNA was mixed with 10 µl of the fragment DNA containing the U2 promoter sequence, and ligated together in a total volume of 25 µl of ligation buffer containing 2 units T4-DNA ligase and 2mM ATP at 14° C overnight. Thereafter, the DNA purified by phenol/chloroform extractions followed by two ethanol precipitations and dissolved in 10 µl distilled water. The resulting sticky ends of EcoRI and Apal had to be converted into blunt ends and ligated. The sticky ends were converted into blunt ends by reaction with the Mung bean nuclease as follows: to 25 µl DNA (1µg/µl concentration) in reaction buffer 20 units of enzyme were added to give a final concentration of 1% glycerol and a final reaction volume of 35 µl. After an incubation for 30 minutes at 30° C the DNA was purified by phenol-chloroform extractions followed by two ethanol precipitations. The DNA was dissolved again in 5 µl of distilled water. The resulting blunt ends were ligated together in 15 µl reaction volume containing 10 x more T4 ligase than used above and 2 mM ATP at 14° C overnight.

The ligation mixture was added to 150 µl competent bacterial cell suspension for DNA up-take. After the DNA up-take the bacterial cells were spread on LB agar plates containing 50 µg/ml ampicillin at volumes of 50 to 300 µl cell suspension per plate. The agar plates were incubated at 37° C overnight. Single isolated bacterial colonies were screened for the presence of a plasmid containing the desired U2-promoter fragment. The resulting plasmid was isolated from the bacterial cells and characterized by restriction enzyme analysis.

7. Ligation of synthetic oligo-DNA-nucleotide 89 (SEQ ID No.: 30) together with MT-promoter fragment (4.5 kb).

The 4.5 kb fragment (described in 1) containing the MT-promoter and a pBR residue were ligated together with the synthetic oligonucleotide 89 (SEQ ID No.:30). The ligation mixture was added to 150 μ l competent bacterial cell suspension for DNA up-take. Single isolated bacterial colonies were screened for the presence of the desired plasmid. The new plasmid was proved by a digestion with the restriction endonucleases EcoRI and XbaI. Two molecules were expected, one 2.0 kb and one 2.6 kb.

8. Ligation of the synthetic oligonucleotide 101 (SEQ ID No.:32) together with plasmid (described in 7).

The plasmid (described in 7) was digested with BglII and BamHI and a fragment of 13 nucleotides was removed (described in 1). The resulting fragment containing the first oligonucleotide 89 (SEQ ID No.:30), was ligated together with oligonucleotide 101 (SEQ ID No.:32), a BglII-BamHI fragment. After DNA up-take single cells were screened for the presence of the desired plasmid. The new plasmid was proved by a digestion with the endonucleases EcoRI and XbaI, or EcoRI and BglII.

9. Ligation of synthetic DNA-oligonucleotide 99 (SEQ ID No.:31) to the 4.5 kb fragment (described in 1).

The 4.5 kb fragment (BglII-BamHI) was ligated together with the DNA oligonucleotide 99 (SEQ ID No.: 31). After screening of single bacterial colonies, containing different DNAs, the desired plasmid was characterized by digestion with EcoRI, resulting in two fragments, 1.9 kb and 2.7 kb, and by positive linearization with BglII or BamHI.

The new plasmid was then digested with PstI and BamHI. Two molecules were expected, one 2.6 kb fragment, containing a pBR residue, the MT-promoter and the oligonucleotide and a 2.0 kb pBR residue. The 2.6 kb fragment was isolated.

10. Ligation of the 2.6 kb fragment of the plasmid described in 9, with a fragment isolated from plasmid (described in 8).

The plasmid (described in 8) containing the DNA oligonucleotides 89 and 101 (SEQ ID No.:30 and 32, respectively) was digested with PstI and BglII. Two fragments were expected. A 2.5kb fragment containing a pBR residue and the MT-promoter and 2.2 kb fragment, containing a pBR residue and both oligos.

This 2.2 kb fragment was ligated together with the 2.6 kb fragment, containing the pBR residue, the MT-promoter and oligo 99 (SEQ ID No.:31) described in 8.

After screening for the desired plasmid, it was characterized by restriction endonuclease digestion with BglII-XbaI. Two fragments were expected, a 270 bp fragment of the oligo-DNA-nucleotides and a 4.5 kb fragment of the MT-promoter and the pBR.

11. Ligation of the 2.3 kb HBV BglII-BglII fragment.

A 2.3 kb BglII-BglII fragment containing the HBV pre-S1, pre-S2 and S coding regions was isolated from HBV-containing DNA. The 2.3 kb fragment was ligated together with the 4.5 kb fragment (obtained as described in 1) containing the methallothionein promoter.

2 μ l of the 2.3 kb fragment was mixed with 3 μ l of the 4.5 kb fragment and ligated together in a total volume of 10 μ l ligation buffer, containing 2 units T4-DNA ligase and 2 mM ATP at 14° C overnight.

The ligation mixture was added to 150 μ l competent bacterial cell suspension for DNA up-take. After the DNA up-take the bacterial cells were spread on LB agar plate containing 50 μ g/ml ampicillin at volumes of 50 to 300 μ l cell suspension per plate. The agar plates were incubated at 37° C overnight. Single isolated bacterial colonies were screened for the presence of a plasmid containing the desired fragment.

12. Conversion of a part of the HBV-gene sequence with HBV -core epitopes.

The plasmid resulting from 11 above was digested with the endonucleases BglII and XbaI. Two molecules were expected, one 550 bp fragment and a 6.25 kb fragment which was isolated after agarose gel electrophoresis.

The 6.25 kb fragment was ligated together with the 270 bp fragment (after digestion with BglII and XbaI and fragment isolation as described above) of the plasmid described in 10, coding for an epitope part of the HBV-core gene.

The ligation mixture was added to 150 μ l competent bacterial cell suspension for DNA up-take. Single isolated bacterial colonies were screened for the presence of the desired plasmid. The new plasmid was proved by a digestion with BamHI. Three molecules were expected, a 950 bp, a 450 bp and a 5,150 bp fragment.

13. Preparation of a "vehicle" plasmid.

The plasmid (described in 11) was digested with EcoRI and XbaI. Two molecules were expected, one 2,450 bp fragment and a 4,350 bp fragment which was isolated after gel electrophoresis.

This 4,350 bp fragment was ligated together with the oligo-DNA-nucleotide 39 (SEQ ID No:29) coding for the entire DNA-sequence of HBV-S-gene from ATG to the XbaI site, wherein the ATG was changed into ATA.

14. Core-epitope upstream of the entire HBV-S gene.

This "vehicle" plasmid was then digested with PstI and XbaI, two molecules were expected, one 600 bp plasmid residue and a 3,850 bp fragment which was isolated and ligated together with a PstI-XbaI fragment of 2,800 bp (2,700 bp) isolated after digestion of the plasmid described in 10.

After screening for the desired plasmid, it was characterized by restriction endonuclease digestion with EcoRI and XbaI, EcoRI and BglII and BamHI.

15. Insertion of a selection marker.

The plasmid (described in 14) was linearized with Eco RI. The reaction was performed in a total volume of 50 μ l and a final concentration of 1 μ g/ μ l plasmid DNA. 50 units of EcoRI were added and the digestion was proved after incubation at 37° C for three hours by agarose gel electrophoresis.

The reaction was stopped by adding 1 μ l of 0.5 M EDTA and DNA was precipitated with a final concentration of 0.3 M sodium acetate and 3-4 volumes of ethanol at -80° C for 30 minutes. The precipitated DNA was dissolved in 50 μ l distilled water.

2 μ l of the linearized plasmid was mixed with 3 μ l of the DNA fragment containing the methallothionein promoter and the neomycin selection gene (described in 4) and ligated together. Single bacterial colonies were screened for the desired plasmid which was isolated, purified and characterized.

Each gene construct described above can be constructed also with the U2-promoter whereby the MT-promoter-containing DNA fragment, after digestion with EcoRI and BglII, is replaced by a DNA fragment containing the U2-promoter isolated after digestion with EcoRI and BglII.

16. Isolation of the E coli xanthine guanine phosphoribosyl transferase (egpt) selection gene.

The fragment containing the egpt selection gene was isolated after digestion of the plasmid pMSG with BamHI and BglII (1.8kb) and ligated together with a 4.5 kb fragment (BglII-BamHI, described in 1) containing the MT-promoter. After screening for the desired plasmid it was isolated, purified and finalized by a conversion of the BamHI site into an EcoRI site.

17. Isolation of desired DNA sequences by PCR-method.

One DNA fragment (400 bp) was isolated after gel electrophoresis. It was generated by PCR-method (described in Example 5) by using the specific oligonucleotides 131 and 132 (SEQ ID No.:33 and 34) as primers.

The DNA fragment was digested with the endonucleases BamHI and XbaI and then purified by gel electrophoresis. The isolated PCR-fragment was ligated together with a 6.25 kb fragment which was isolated from the plasmid (described in 13) after digestion with BglII and XbaI. After DNA up-take and bacterial transformation the single bacterial colonies were screened for the desired plasmid.

18. Insertion of a selection marker.

The plasmid (described in 17) was finalized by adding a selection gene to the plasmid (described in 15).

19. Isolation of the H2K promoter.

The H2K-promoter was isolated as an EcoRI and BglII fragment (2kb) from pSP65H2 (available from Exogene).

In all constructs described all promoters are replaceable as EcoRI/BglII fragments.

20. Conversion of a part of the HBV-gene sequence.

The plasmid resulting from 11) above was digested with the endonucleases BglII and XbaI. Two molecules were expected, one of which is a 6.250 kb fragment which was isolated after agarose gel electrophoresis.

The 6.250 kb fragment was ligated together with oligo-DNA-nucleotide 23 (SEQ ID No.:28). The ligation mixture was added to 150 μ l competent bacterial cell suspension for DNA up-take. Single isolated bacterial colonies were screened for the presence of the desired plasmid. The new plasmid was proven by a digestion with the endonucleases EcoRI and BglII. Two molecules were expected, one 1.9 kb and one 4.450 kb.

21. Insertion of a egpt selection marker.

The plasmid (described in 20) was linearized with EcoRI. The reaction was performed in a total volume of 100 μ l and a final concentration of 0.6 μ g/ μ l plasmid DNA. 60 units of EcoRI were added and the digestion was proved after incubation at 37° C for three hours by agarose gel electrophoresis. The reaction was stopped by adding 2 μ l of 0.5 M EDTA and the DNA was precipitated with a final concentration of 0.3 M sodium acetate and 4 volumes of ethanol at -80° C for 1 hour. The precipitated DNA was dissolved in 50 μ l distilled water.

2 μ l of the linearized plasmid was mixed with 3 μ l of the DNA-fragment (3.7 kb) containing the methallothionein promoter and the egpt selection gene (described in 16) by digestion with EcoRI and ligated together. Single colonies were screened for the presence of the desired plasmid. Each of the

described gene constructs in Table III are preparable in the same way as described above.

Example 4

5 Transfection of Mammalian Cells with Constructs of the Present Invention.

In order to achieve secretion of substantial amounts of the HBV peptides encoded by constructs of the present invention, mammalian cells must be transfected with a DNA construct of the present invention. The cotransfection was performed in two steps (i.e. a separate transfection for each construct) or in a single step
10 (i.e. one transfection using preparation of both constructs). Cotransfection was confirmed either by use of different selection markers on the two constructs or by detection of secretion of expression products of both constructs by immunoassay.

Alternatively, a sequence encoding the HBV peptide sequence of the present invention and a separate sequence encoding the entire S or core or HAV protein could be combined in a single construct.

15 Example 5

Polymerase chain reaction (PCR).

20 The polymerase chain reaction allows to amplify specific DNA nucleotide sequences of a selected region of a known genomic sequence in vitro by more than a millionfold (Thomas J. White, Norman Arnheim, Henry A. Erlich 1989: The polymerase chain reaction. Technical Focus, Vol. 5. No. 6; S. Kwok and R. Higuchi 1989: Avoiding false positives with PCR. Nature, Vol. 339, pp 237-238).

DNA isolated from cells or plasmid DNA is treated to separate its complementary strands. These
25 strands are then annealed with an excess of two DNA oligonucleotides (each 20 - 25 base pairs long) that have been chemically synthesized to match sequences separated by X nucleotides (where X is generally between 50 to 2,000 base pairs).

The two oligonucleotides serve as specific primers for in vitro DNA synthesis catalysed by DNA polymerase which copies the DNA between the sequences corresponding to the two oligonucleotides. If the
30 two primer oligonucleotides contain the correct sequence it is possible to create new digestion sites at the 5' and 3'.

After multiple cycles of reaction, a large amount of a DNA fragment of the desired length was obtained, purified by gel electrophoresis and characterized by restriction enzyme digestion and agarose gel elec-
trophoresis. The amplified, purified DNA fragment was then used to ligate it together with other fragments
35 i.e. plasmid.

The PCR-DNA fragments were amplified with blunt end. To get sticky end (for the ligation procedure) the fragment has to be digested with the desired endonucleases and purified again.

The PCR-reaction will work for 20 to 30 cycles. One cycle is separated into three steps with different reaction times and different reaction temperatures which is controlled by a PCR-thermo-cycler. The first
40 step is "Denaturation" of the matrix-DNA (1 min/95° C), the second step is "Hybridisation" of matrix DNA and primers (1 min/55° C) followed by "Polymerisation" (2 min/72° C).

The final volume for one assay is 30 µl for example, which contains the following final concentrations: PCR-buffer (1 x), nucleotide-mix with 200 µM of each of the four nucleotides, 200ng for 30 µl of each of the
two primers, 0.5 units Taq-Polymerase per 30 µl aqua bidest.

45 Example 6

Culturing of Transfected Cells to Secrete Protein

50 The recipient cells (C127 or CHO-cells available from ATCC) were seeded in normal growth medium (DMEM + 10% Fetal Calf Serum, Glucose and Glutamine) into petri-dishes (1-2 x 10⁶ cells per dish, ϕ 10 cm) at day 1. The next day the medium was removed (4 hours before the DNA precipitate was added onto the cells), and the cells were washed twice with 1 x PBS. Then 8 ml DMEM without FCS were added, 4 hours later the DNA precipitate (prepared as described below) was added to the cells. Again after 4 hours
55 the medium was removed, 3 ml of Glycerol-Mix (50 ml 2 x TBS buffer, 30 ml glycerol, 120 ml distilled water) were added. The Glycerol-Mix was immediately removed after an incubation at 37° C for 3 minutes and the cells were washed with 1 x PBS. The cells were cultivated overnight with 8 ml of DMEM with 10% FCS.

After 48 hours, the cells were recovered from the dish by treating with Trypsin-EDTA-Solution (0.025% Trypsin + 1mM EDTA). Afterwards, to remove the Trypsin-EDTA the cells were washed with 1 x PBS, suspended in DMEM with 10% FCS and distributed into 24 costar-well-plates (cells from one dish into four 24-well-plates).

- 5 When the cells had grown well, selection medium was added (concentration 0.5 - 1 mg/ml of neomycin or: xanthine (250 μ g/ml), hypoxanthine (15 μ g/ml) or adenine (25 μ g/ml), thymidine (10 μ g/ml), aminopterin (2 μ g/ml), mycophenolic acid (25 μ g/ml) for eco-gpt, for example). The medium was changed every week. The first growing cell colonies were seen after 2 weeks.

- 10 To 10 μ g of plasmid DNA and 20 μ g of carrier-DNA (salmon sperm DNA, calf-thymus DNA) TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.05) was added to a final volume of 440 μ l and mixed together with 60 μ l 2 M CaCl_2 . Then the same amount of 2x TBS (Hepes 50 mM, NaCl 280 mM, Na_2HPO_4 1.5 mM, pH 7.05) was added and mixed well. The precipitation solution was incubated for 30 minutes at 37° C and added directly to the cells which were to be transfected.

15 Example 7

Preparation of the Adjuvant of Purified Particles.

- 20 To the desired concentration of antigen suspended in sterile saline, 1 : 10,000 volume Thimerosal, 1/10 volume of filter-sterilized 0.2 M $\text{KAl}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ were added. The pH was adjusted to 5.0 with sterile 1 N NaOH and the suspension was stirred at room temperature for 3 hours. The alum-precipitated antigen was recovered by centrifugation for 10 minutes at 2,000 rpm, resuspended in sterile normal saline containing 1:10,000 Thimerosal and aliquoted under sterile conditions.

25 Example 8

Purification of Hepatitis-B-core Antigen.

- 30 The cell supernatant of HB-core-antigen-secreting cells was collected and concentrated by ultrafiltration. The concentrate was cleared by centrifugation at 20,000 rpm for 15 minutes at 4° C in a Beckman SW28 rotor.

Particle formation was tested by sucrose density centrifugation (0-45% sucrose) in a Beckman SW28 rotor for 24 hours at 28,000 rpm and 4° C. The gradient was fractionated and the single fractions were analyzed by Elisa.

35 Example 9

The following tables give some results of Elisa analysis of immunogenic particles of the present invention as described below:

- 40 Table IV shows the Elisa data of the purified HBs-antigen particle produced from any HBV-sequence construct of the present invention including the pre-S1 epitopes and the S region with the anti-pre-S1 monoclonal antibody MA 18/7 and the anti-HBs monoclonal antibody G022.

Table IV shows the fractions (21) collected after CsCl density gradient.

45 Table IV-1

CsCl-gradient Fraction No.	Elisa Measurement (E = 492) Monoclonal Antibody 18/7
13	0.092
14	0.210
15	0.388
16	1.662
17	2.604
18	0.648
19	0.031

Table IV-2

CsCl-gradient Fraction No.	Elisa Measurement (E = 492) Monoclonal Antibody G022
13	0.136
14	0.426
15	0.822
16	1.970
17	2.954
18	0.967
19	0.076

Table V shows the Elisa data of the purified HB-core-antigen particles produced from any HB-core-sequence construct of the present invention with polyclonal antibodies against HB-core and with monoclonal antibody G022 against HB-S-Ag.

Table V-1

Sucrose Gradient Fraction No.	Elisa Measurement (E = 492) Polyclonal Antibodies
6	0.25
7	0.922
8	1.423
9	1.5
10	1.5
11	1.28
12	0.466

Table V-2

Sucrose Gradient Fraction No.	Elisa Measurement (E = 492) Monoclonal Antibody G022
6	0.020
7	0.024
8	0.018
9	0.011
10	0.015
11	0.020
12	0.022

Example 10

Studies of administering Hepa-Care in Chimpanzees:

Hepa-Care are particles presenting hepatitis B surface antigens (S1 and S) in a specific formulation (ratio 50:50), which are used for the treatment of chronic carriers of hepatitis virus.

Experiment 1

A Hepatitis-B-carrier chimpanzee 1 was treated (intramuscularly) with Hepa-Care at time 0, 4, and 8 weeks with a dosage of 18 μ g per injection.

The liver enzymes were monitored (Fig. IV) as well as the hepatitis-B antigen level (Fig. V).

Experiment 2

Chimpanzee 1 after treatment described above was given a booster treatment at week 30, 34, and 38. The results are shown in Fig. VI.

Experiment 3

Chimpanzee 2 was treated with Hepa-Care, but contrary to chimpanzee 1 it was given intravenously. The dosage was 2 mg. The results are shown in Fig. VII.

From a control chimpanzee 3 the liver enzymes were also monitored and shown in Fig. VIII.

Example 11

Treatment with Hepa-Care:

(for definition see Example 10)

Patient 1 (male, age = 65 years, disease for 2 years): Hepatitis-B parameters:

HBsAg pos.

anti-HBs neg.

HBeAg neg.

anti-HBe pos.

anti-HBc neg.

was treated (i.m.) with Hepa-Care at month 0, 1, 6, and 7. The results of the antigen and antibody measurements are given in Fig. IX and X.

Patient 2 (female, age = 48 years, disease for 12 years):

Hepatitis-B parameters:

HBsAg pos.

HBeAg neg.

anti-HBs neg.

anti-HBe pos.

anti-HBc pos.

was treated (i.m.) with Hepa-Care at month 0, 1, and 6. Results of antigen and antibody measurements are shown in Fig. XI and XII.

Patient 3 (female, age = 41 years, disease for 5 years):

Hepatitis-B parameters:

HBsAg pos.

HBeAg neg.

anti-HBs neg.

anti-HBe pos.

was treated at month 0, 1, 2, and 5 with Hepa-Care (i.m.). The measured values of HBs antigen and anti-HBs antibodies are shown in Fig. XIII and XIV.

SEQUENCE LISTING

5
 10
 15
 20
 25
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 40
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 50
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SEQ ID NO: 1
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 558 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

25
 30
 35
 40
 45
 50
 55

ATG	GAC	ATT	GAC	CCT	TAT	AAA	GAA	TTT	GGA	GCT
ACT	GTG	GAG	TTA	CTC	TCG	TTT	TTG	CCT	TCT	GAC
TTC	TTT	CCT	TCC	GTA	CGA	GAT	CTC	CTA	GAC	ACC
GCC	TCA	GCT	CTG	TAT	CGA	GAA	GCC	TTA	GAG	TCT
CCT	GAG	CAT	TGC	TCA	CCT	CAC	CAT	ACT	GCA	CTC
AGG	CAA	GCC	ATT	CTC	TGC	TGG	GGG	GAA	TTG	ATG
ACT	CTA	GCT	ACC	TGG	GTG	GGT	AAT	AAT	TTG	CAA
GAT	CCA	GCA	TCC	AGA	GAT	CTA	GTA	GTC	AAT	TAT
GTT	AAT	ACT	AAC	ATG	GGT	TTA	AAG	ATC	AGG	CAA
CTA	TTG	TGG	TTT	CAT	ATA	TCT	TGC	CTT	ACT	TTT
GGA	AGA	GAG	ACT	GTA	CTT	GAA	TAT	TTG	GTC	TCT
TTC	GGA	GTG	TGG	ATT	CGC	ACT	CCT	CCA	GCC	TAT
AGA	CCA	CCA	AAT	GCC	CCT	ATG	TTA	TCA	ACA	CTT
CCG	GAA	ACT	ACT	GTT	GTT	AGA	CGA	CGG	GAC	CGA
GGC	AGG	TCC	CCT	AGA	AGA	AGA	ACT	CCC	TCG	CCT
CGC	AGA	CGT	AGA	TCT	CAA	TCG	CCG	CGT	CGC	AGA
AGA	TCT	CAA	TCT	CGG	GAA	TCT	CAA	TGT	TAG	

SEQ ID NO: 2
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 504 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

```

20      ATG  GAC  ATT  GAC  CCT  TAT  AAA  GAA  TTT  GGA  GCT
      ACT  GTG  GAG  TTA  CTC  TCG  TTT  TTG  CCT  TCT  GAC
25      TTC  TTT  CCT  TCC  GTA  CGA  GAT  CTC  CTA  GAC  ACC
      GCC  TCA  GCT  CTG  TAT  CGA  GAA  GCC  TTA  GAG  TCT
      CCT  GAG  CAT  TGC  TCA  CCT  CAC  CAT  ACT  GCA  CTC
30      AGG  CAA  GCC  ATT  CTC  TGC  TGG  GGG  GAA  TTG  ATG
      ACT  CTA  GCT  ACC  TGG  GTG  GGT  AAT  AAT  TTG  CAA
      GAT  CCA  GCA  TCC  AGA  GAT  CTA  GTA  GTC  AAT  TAT
35      GTT  AAT  ACT  AAC  ATG  GGT  TTA  AAG  ATC  AGG  CAA
      CTA  TTG  TGG  TTT  CAT  ATA  TCT  TGC  CTT  ACT  TTT
      GGA  AGA  GAG  ACT  GTA  CTT  GAA  TAT  TTG  GTC  TCT
40      TTC  GGA  GTG  TGG  ATT  CGC  ACT  CCT  CCA  GCC  TAT
      AGA  CCA  CCA  AAT  GCC  CCT  ATG  TTA  TCA  ACA  CTT
45      CCG  GAA  ACT  ACT  GTT  GTT  AGA  CGA  CGG  GAC  CGA
      GGC  AGG  TCC  CCT  AGA  AGA  AGA  ACT  CCC  TCG  CCT
50      CGC  AGA  CGT

```

SEQ ID NO: 3
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 504 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

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20      ATG  GAC  ATT  GAC  CCT  TAT  AAA  GAA  TTT  GGA  GCT
      ACT  GTG  GAG  TTA  CTC  TCG  TTT  TTG  CCT  TCT  GAC
25      TTC  TTT  CCT  TCC  GTA  CGA  BAT  CTC  CTA  GAC  ACC
      GCC  TCA  GGT  CTG  TAT  CGA  GAA  GCC  TTA  GAG  TCT
      CCT  GAG  CAT  TGC  TCA  CCT  CAC  CAT  ACT  GCA  CTC
30      AGG  CAA  GCC  ATT  CTC  TGC  TGG  GGG  GAA  TTG  ATG
      ACT  CTA  GCT  ACC  TGG  GTG  GGT  AAT  AAT  TTG  CAA
      GAT  CCA  GCA  TCC  AGA  GAT  CTA  GTA  GTC  AAT  TAT
35      GTT  AAT  ACT  AAC  ATG  GGT  TTA  AAG  ATC  AGG  CAA
      CTA  TTG  TGG  TTT  CAT  ATA  TCT  TGC  CTT  ACT  TTT
40      GGA  AGA  GAG  ACT  GTA  CTT  GAA  TAT  TTG  GTC  TCT
      TTC  GGA  GTG  TGG  ATT  CGC  ACT  CCT  CCA  GCC  TAT
      AGA  CCA  CCA  AAT  GCC  CCT  ATG  TTA  TCA  ACA  CTT
45      CCG  GAA  ACT  ACT  GTT  GTT  AGA  CGA  CGG  GAC  CGA
      GGC  AGG  TCC  CCT  AGA  AGA  AGA  ACT  CCC  TCG  CCT
50      CGC  AGA  CGT
  
```


Fig. XIV

ANTIBODY MEASUREMENT

PATIENT #3

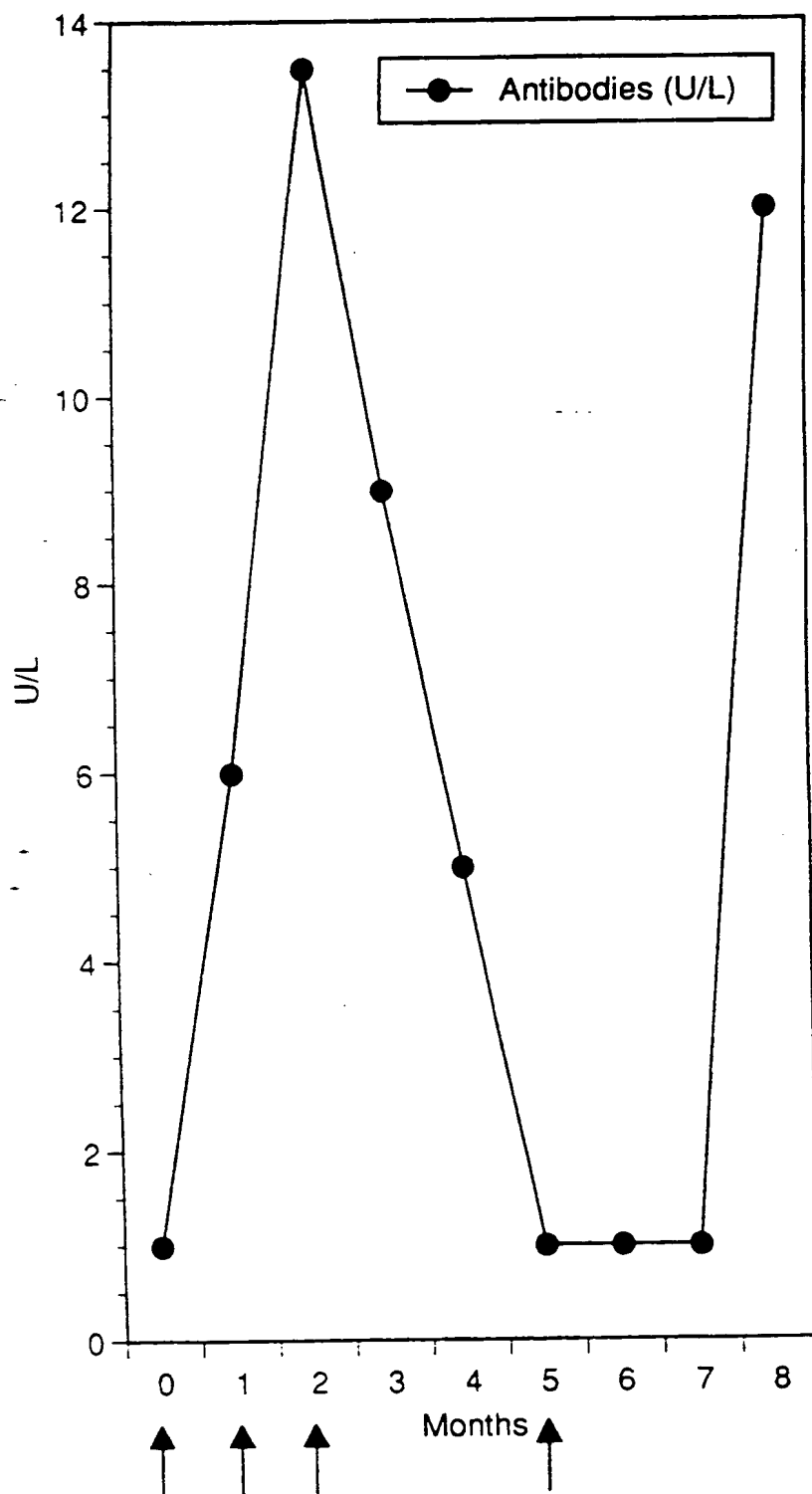


Fig. XIII **ANTIGEN MEASUREMENT**
PATIENT #3

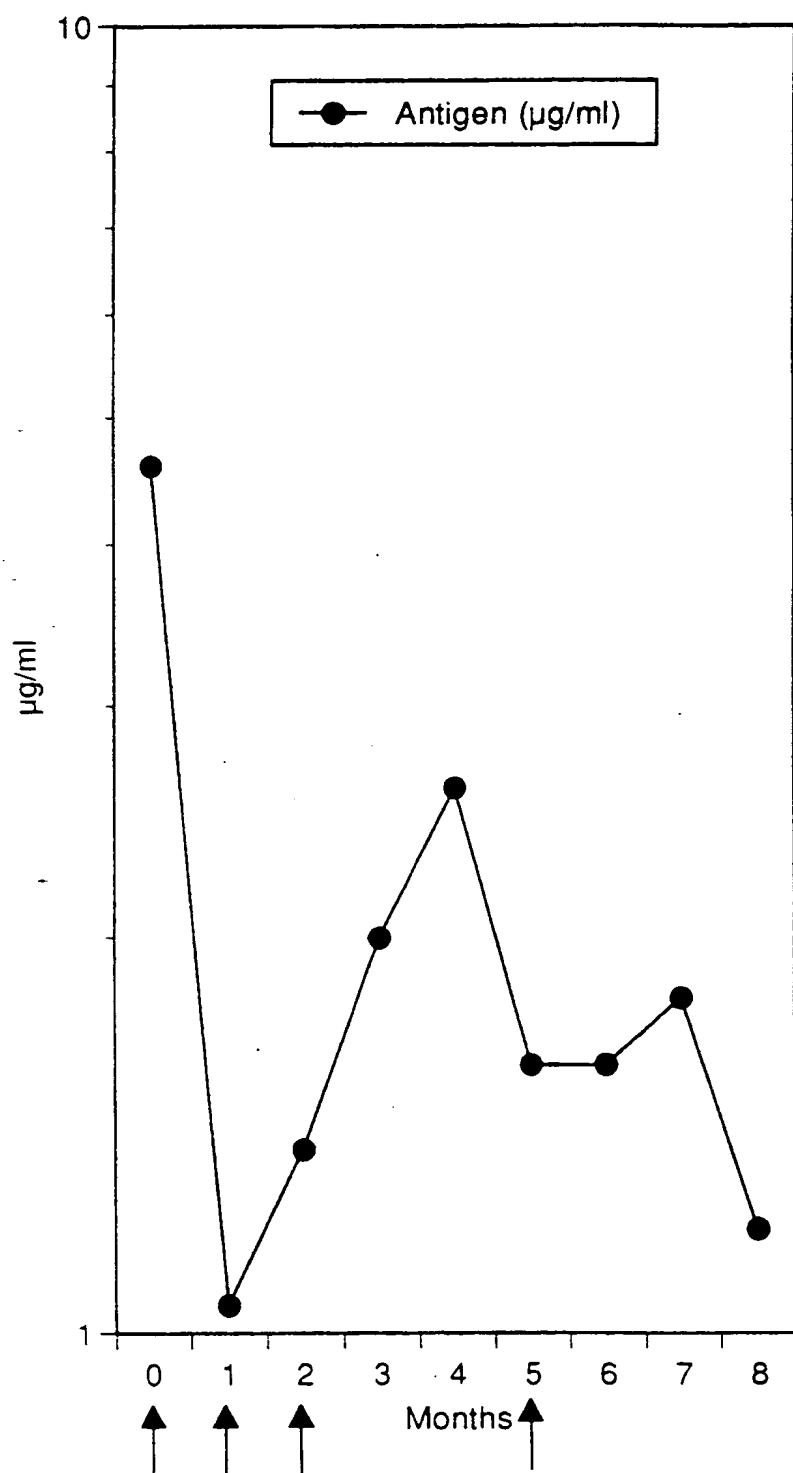


Fig. XII

ANTIBODY MEASUREMENT

PATIENT #2

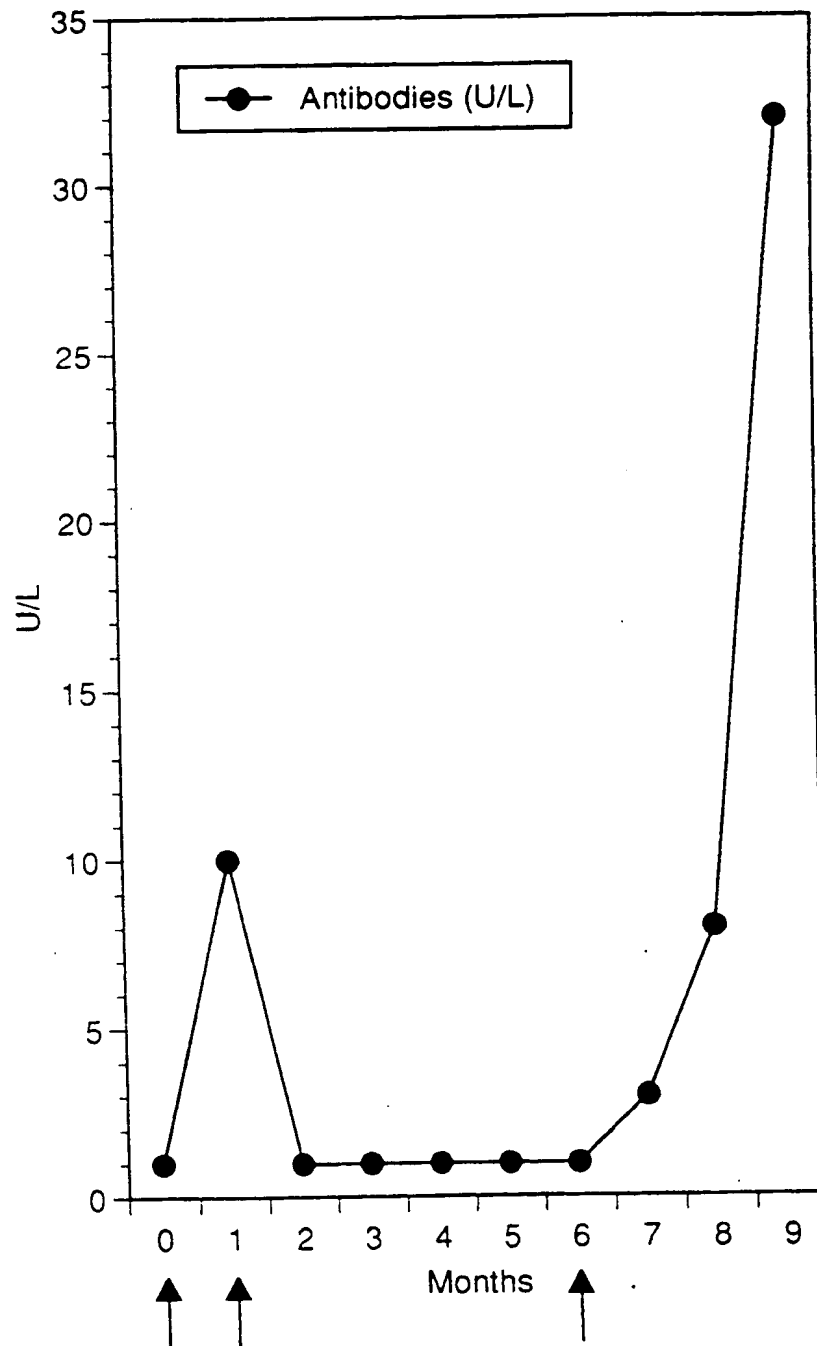


Fig. XI **ANTIGEN MEASUREMENT**
PATIENT #2

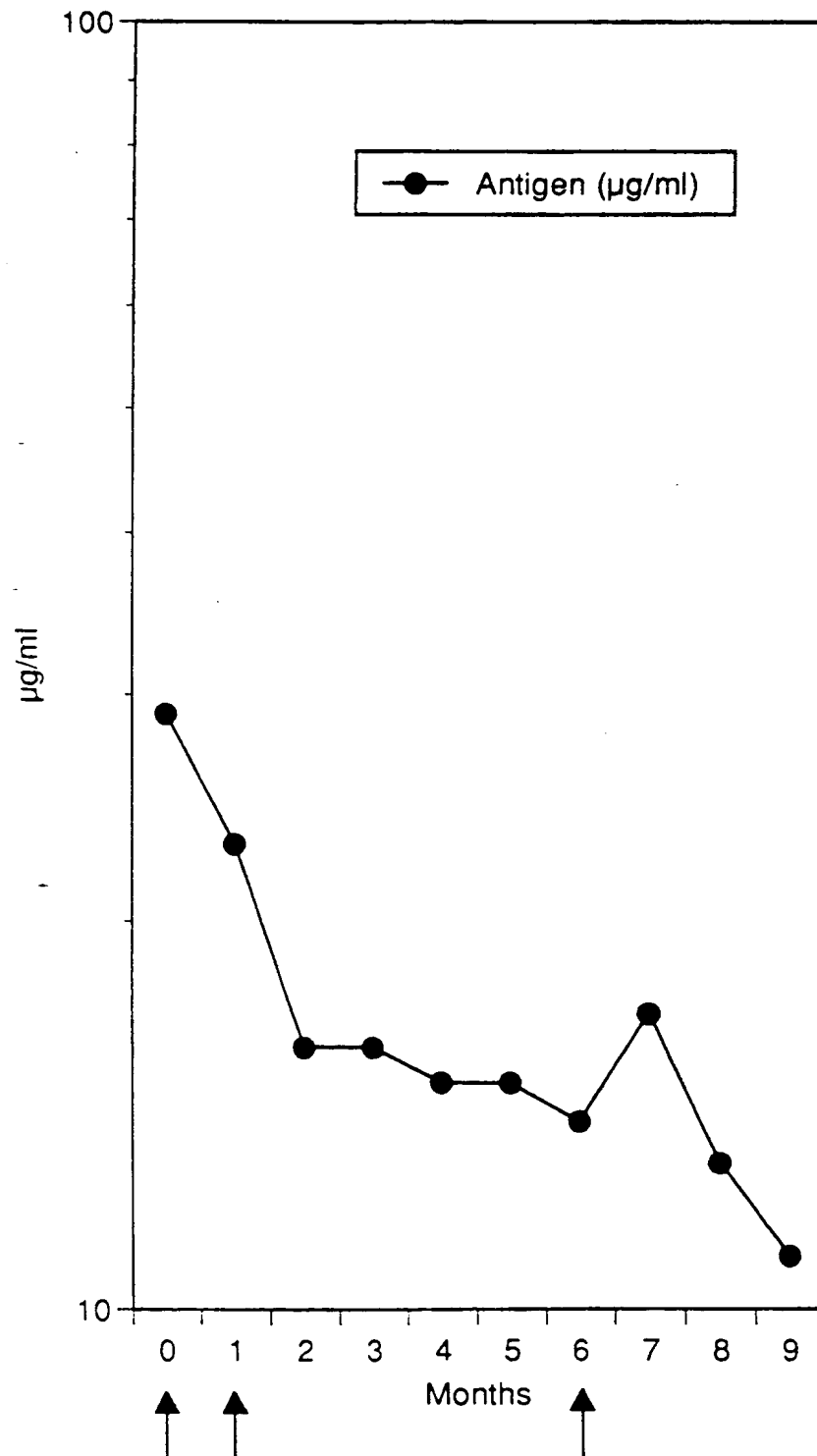


Fig. X

ANTIBODY MEASUREMENT

PATIENT #1

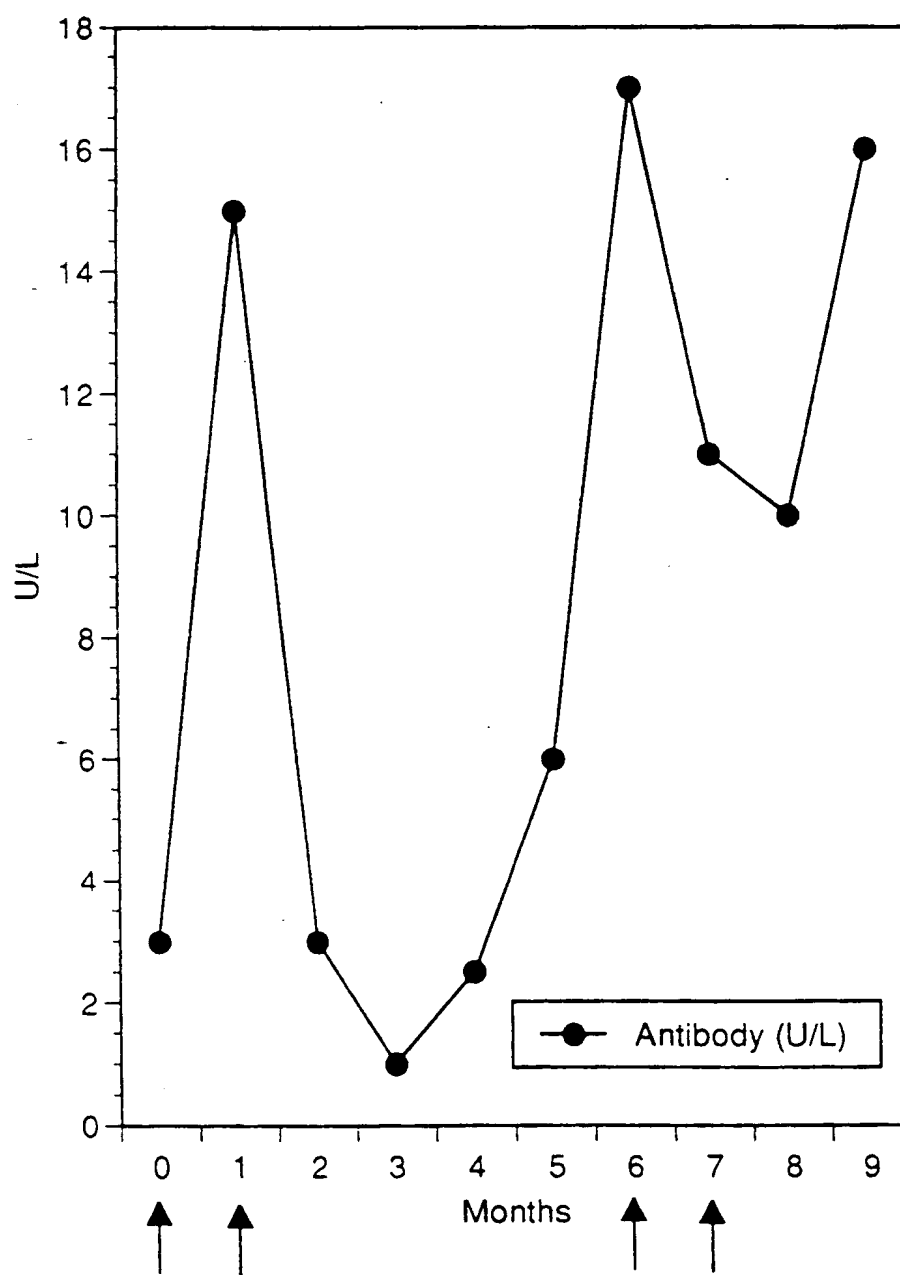


Fig. IX

ANTIGEN MEASUREMENT

PATIENT #1

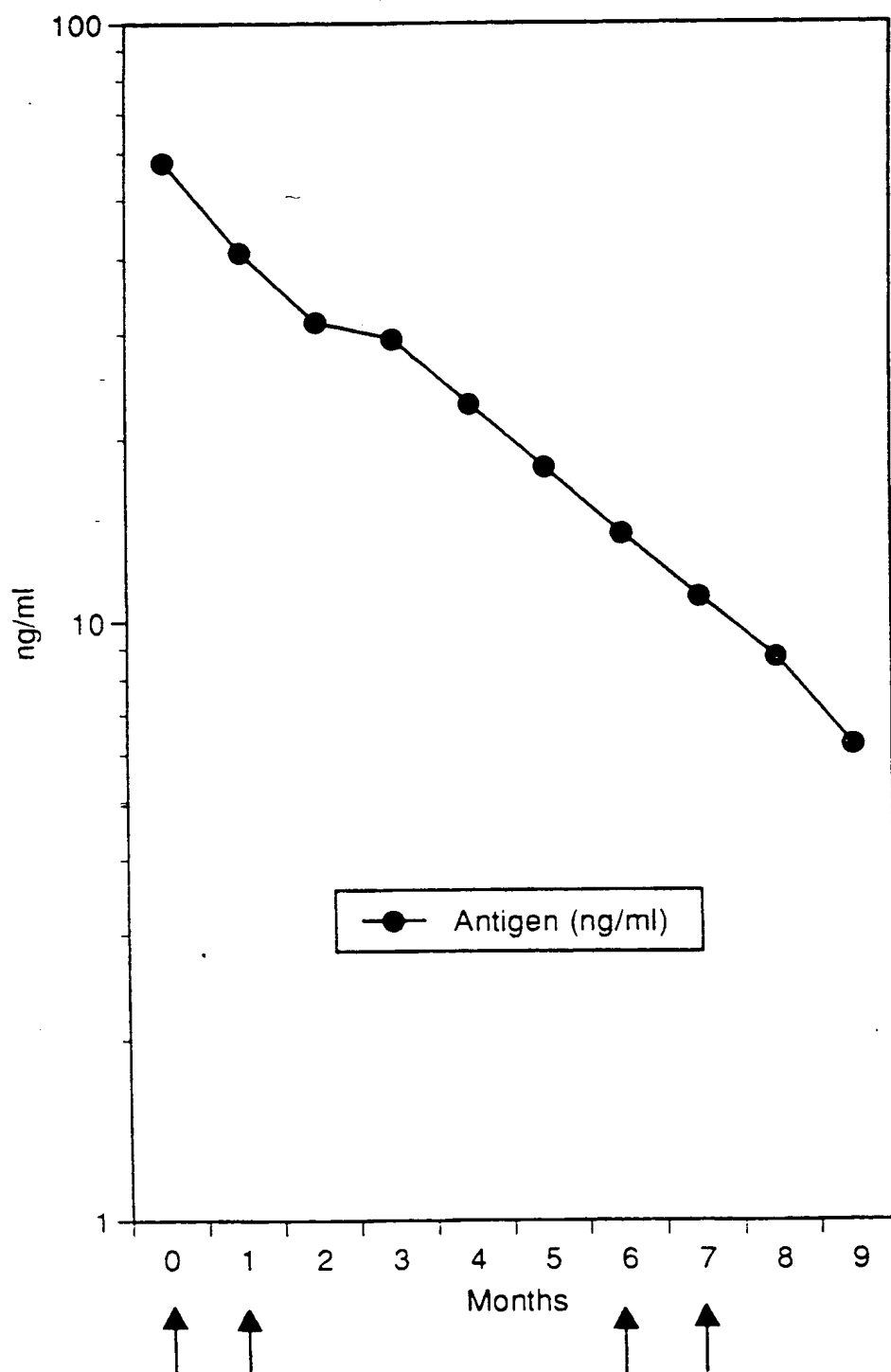


Fig. VIII Measurement of Liver Enzymes of a Control Chimpanzee without Treatment

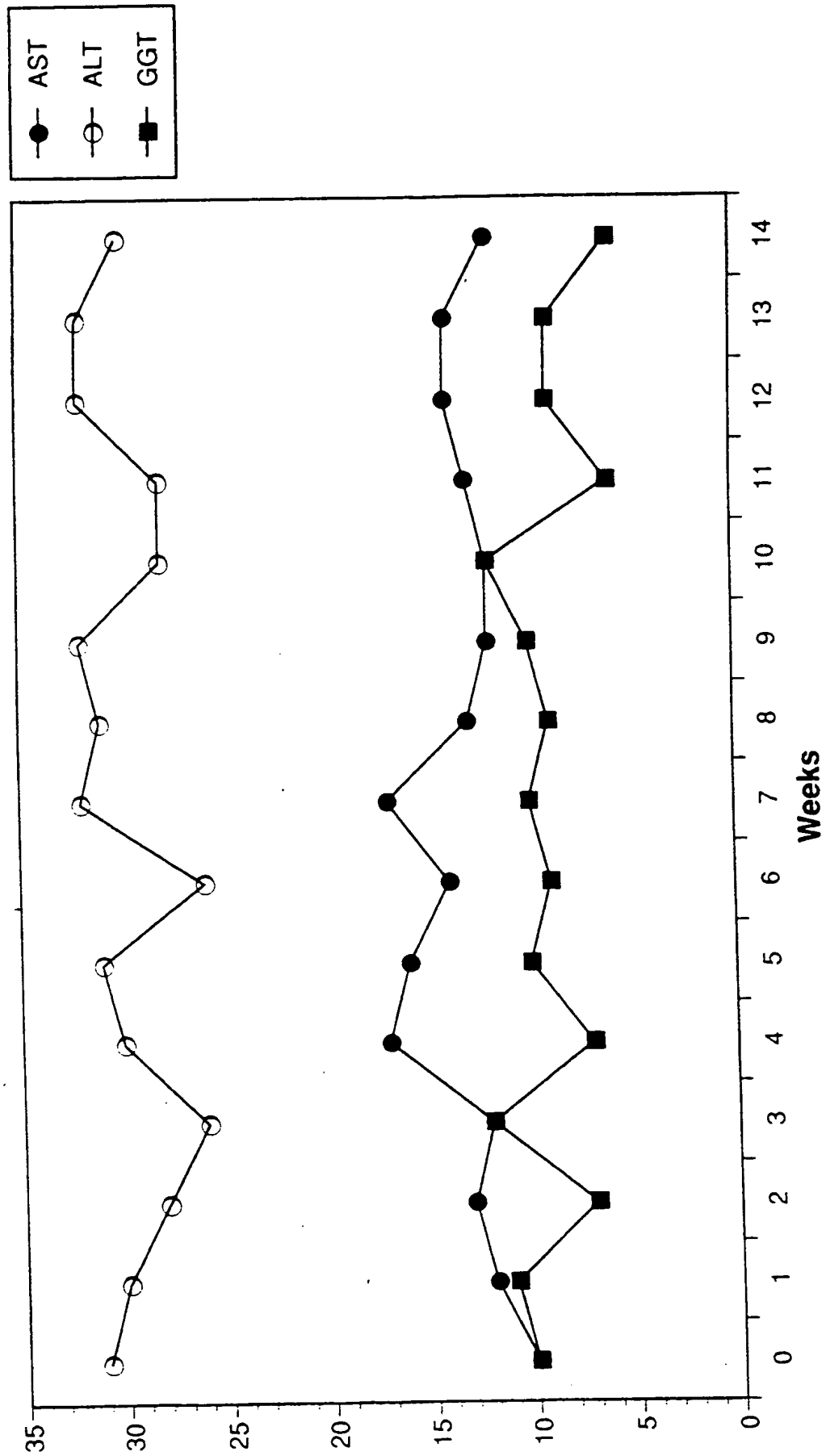


Fig. VII

RESULTS OF CHIMPANZEE EXPERIMENT 3

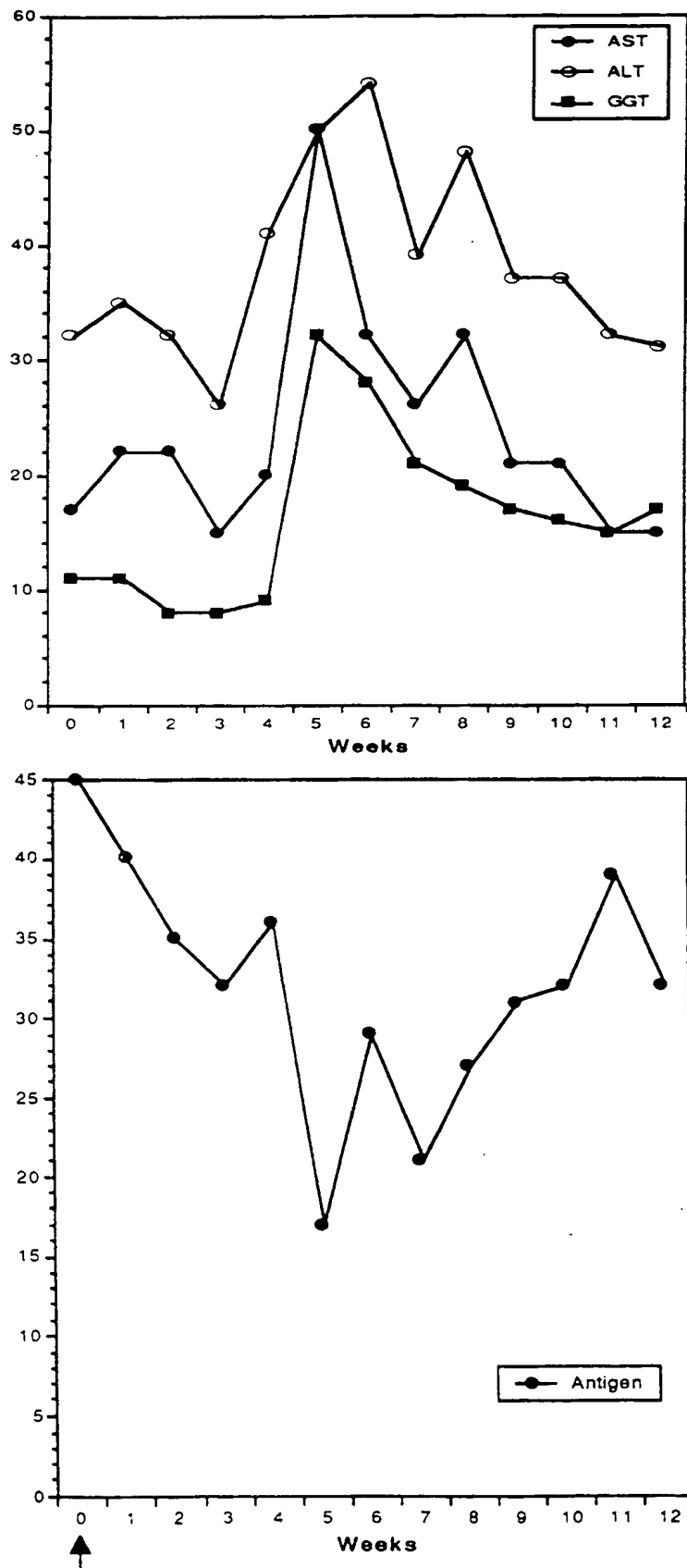


Fig. VI **LIVER ENZYME MEASUREMENT**
CHIMPANZEE EXPERIMENT 2

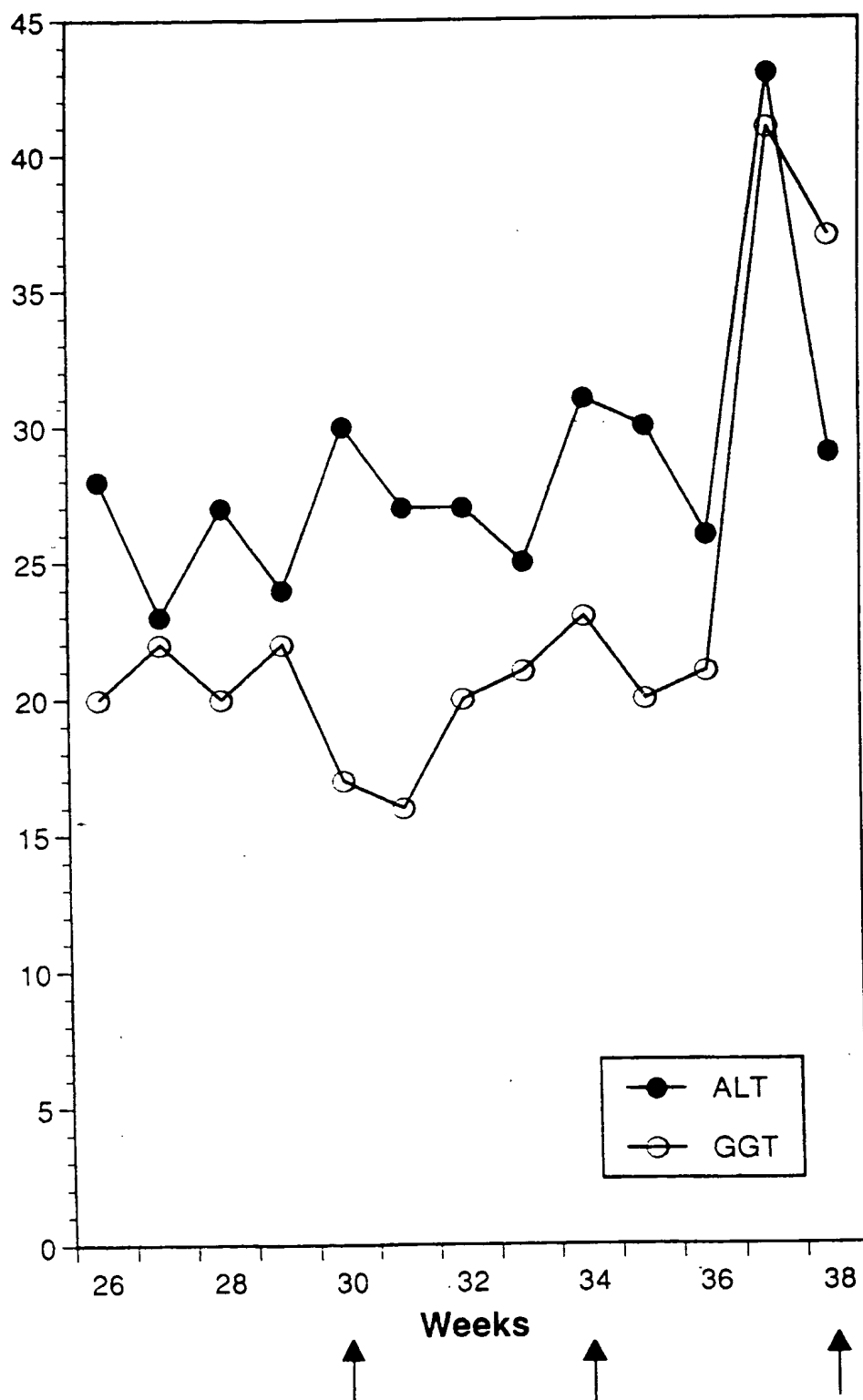


FIG. V **ANTIGEN MEASUREMENT**
CHIMPANZEE EXPERIMENT 1

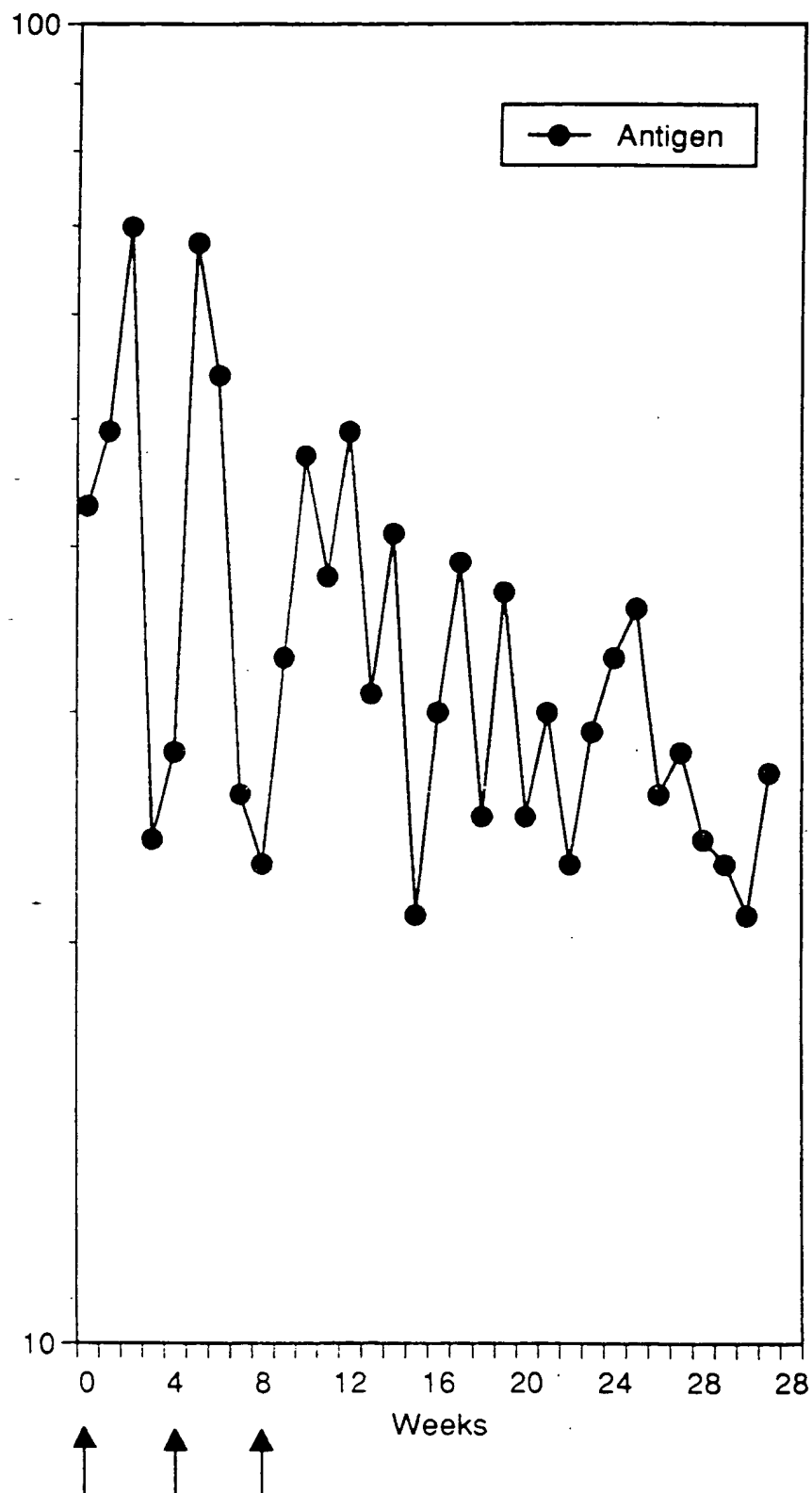


Fig. IV

LIVER ENZYME MEASUREMENT

CHIMPANZEE EXPERIMENT 1

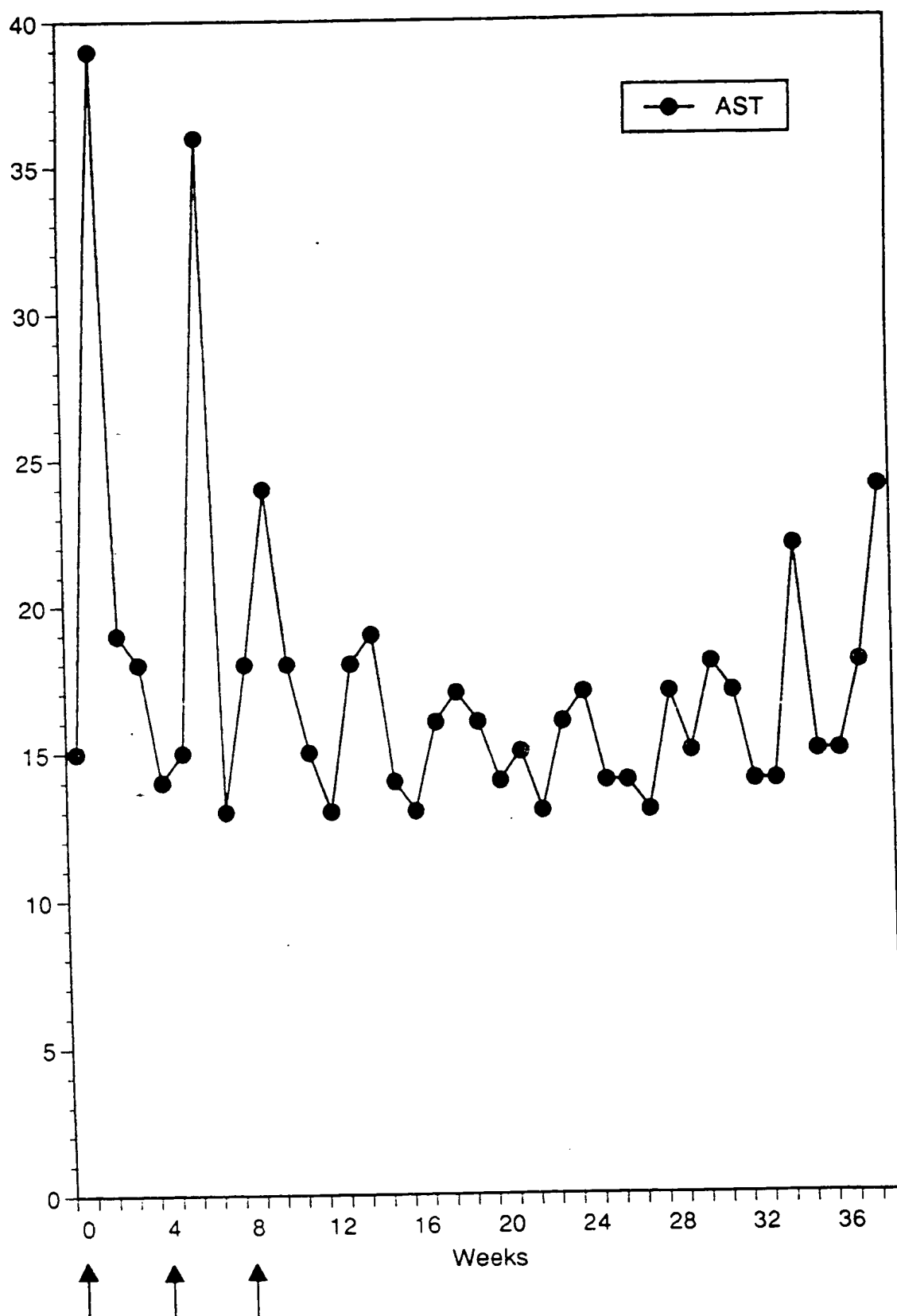


Fig. III shows a DNA construct presenting a promoter, a T-cell epitope with a particle former residue and a selection gene (described in Example 3/21)

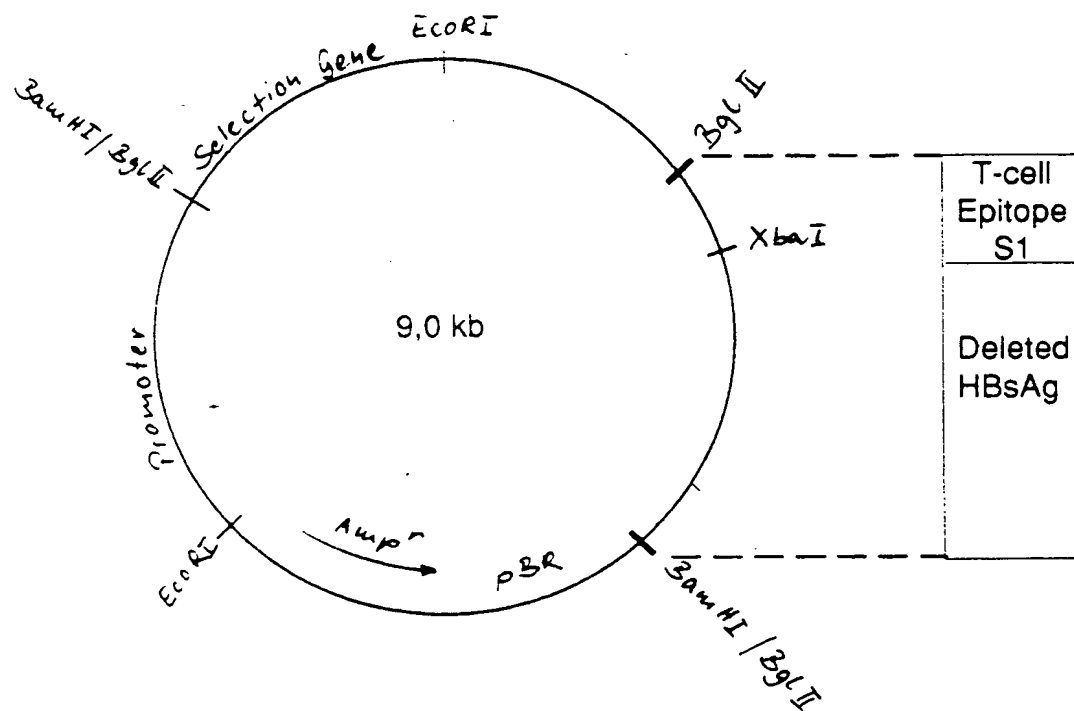


Fig. II shows a DNA-gene construct containing a promoter, an epitope with the entire HB-S-Ag and a selection gene (described in Example 3/18)

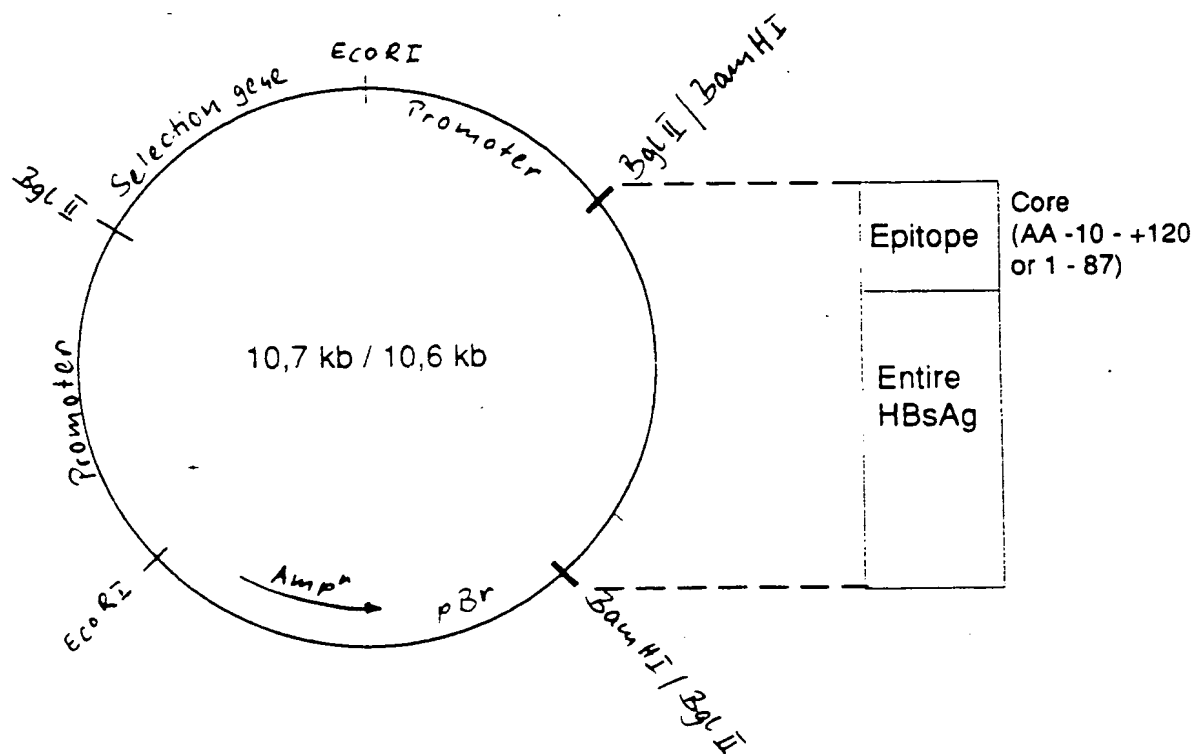
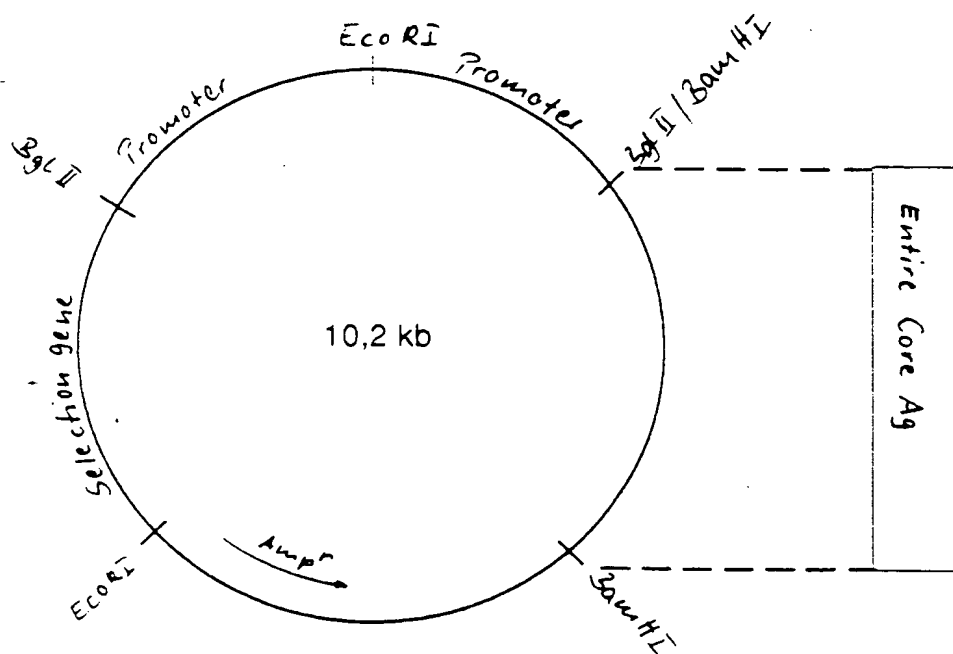


Fig. 1

shows a DNA construct, coding for a promoter, a particle former sequence and a selection gene (described in Example 3/4)



16. Use of a combination according to any of the claims 1 to 15 for the production of a medicament for the treatment of chronic viral hepatitis.

17. A recombinant DNA molecule coding for a combination according to any of the claims 1 to 16 comprising at least one first DNA sequence, optionally a second, a third and/or a fourth DNA sequence, wherein

i) said at least one first DNA sequence codes for at least one polypeptide sequence a) according to one of claims 1 to 6.

ii) said second DNA sequence codes for a polypeptide sequence b) according to one of claims 8 to 12,

iii) said third DNA sequence codes for a spacer sequence according to claim 15, and

iv) said fourth DNA sequence codes for a selection marker,

and wherein the DNA sequences are controlled by DNA elements essential for expression, and optionally have a common reading frame.

18. A recombinant DNA molecule according to claim 17, characterised in that in the first DNA sequence(s) up to 30% nucleotides may be substituted.

19. A host cell transfected with a recombinant DNA molecule according to either of claims 17 or 18.

20. A host cell according to claim 19, characterised in that said host cell is selected from the group comprising mammalian, yeast and bacterial cells.

a) can be bound to carrier b) by adsorption, any chemical bonding or secondary valences.

2. A combination according to claim 1, characterised in that polypeptide sequence(s) a) mediate(s) the antigenicity of a T cell-activating epitope in direct or indirect way.
- 5 3. A combination according to claim 1 or 2, characterised in that said polypeptide sequence(s) a) is a polypeptide of hepatitis B virus.
- 10 4. A combination according to one of claims 1 to 3, characterised in that polypeptide sequence(s) a) is the amino acid sequence of one or more members selected from the group comprising the HB viral peptides pre S1, pre S2, S and the core antigens.
- 15 5. A combination according to one of claims 1 to 4, characterised in that the polypeptide sequence(s) a) may be modified:
 - i) by having arbitrary deletions, whereby an epitope comprising at least six consecutive amino acid residues is preserved,
 - ii) by having substitutions of one or several amino acids, or
 - iii) by carrying an additional amino acid sequence either at its N-terminus, at its C-terminus or as an insertion into the polypeptide sequence(s) a).
- 20 6. A combination according to one of claims 1 to 5, characterised in that polypeptide sequence(s) a) is myristylated.
- 25 7. A combination according to one of claims 1 to 6, characterised in that the carrier b) is a polysaccharide, a hydrophobic polymer or an inorganic molecule having particle form.
8. A combination according to one of claims 1 to 6, characterised in that the carrier b) is a second polypeptide sequence.
- 30 9. A combination according to claim 8, characterised in that polypeptide sequence b) upon secretion forms particles having a diameter of at least 10nm.
- 35 10. A combination according to claim 8 or 9, characterised in that polypeptide sequence b) is a substantial part of or the complete amino acid sequence of a polypeptide selected from a group comprising the HBV S-peptide, the HBV core-, the HAV core- and the HIV core-antigen as well as the surface antigens of poliovirus, HAV or HIV.
- 40 11. A combination according to one of claims 8 to 10, characterised in that polypeptide sequence b) may be modified
 - i) by having arbitrary deletions, whereby the particle forming capacity is preserved,
 - ii) by having substitutions of one or several amino acids, or
 - iii) by carrying an additional amino acid sequence either at its N-terminus, at its C-terminus or as an insertion into the polypeptide sequence b).
- 45 12. A combination according to one of claims 8 to 11, characterised in that polypeptide sequence b) is myristylated.
13. A combination according to one of claims 1 to 6 or 8 to 12, characterised in that the polypeptide sequences a) and b) are linked via disulfide bridges.
- 50 14. A combination according to one of claims 1 to 6 or 8 to 13, characterised in that the polypeptide sequences a) and b) are linked via "hydrophobic anchoring" (mediated by myristic acid).
- 55 15. A combination according to one of claims 1 to 6 and 8 to 14, characterised in that the polypeptide sequences a) and b) are linked by a peptide bond, wherein optionally a spacer sequence is inserted in between polypeptide sequence(s) a) and polypeptide sequence b), said spacer sequence being linked via peptide bonds to polypeptide sequences a) and b).

SEQ ID NO: 35
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 588 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

	TCC	AAC	CTG	TGC	CTT	GGG	TGG	CTT	TGG	GGC	
20	ATG	GAC	ATT	GAC	CCT	TAT	AAA	GAA	TTT	GGA	GCT
	ACT	GTG	GAG	TTA	CTC	TCG	TTT	TTG	CCT	TCT	GAC
	TTC	TTT	CCT	TCC	GTA	CGA	GAT	CTC	CTA	GAC	ACC
25	GCC	TCA	GCT	CTG	TAT	CGA	GAA	GCC	TTA	GAG	TCT
	CCT	GAG	CAT	TGC	TCA	CCT	CAC	CAT	ACT	GCA	CTC
	AGG	CAA	GCC	ATT	CTC	TGC	TGG	GGG	GAA	TTG	ATG
30	ACT	CTA	GCT	ACC	TGG	GTG	GGT	AAT	AAT	TTG	CAA
	GAT	CCA	GCA	TCC	AGA	GAT	CTA	GTA	GTC	AAT	TAT
35	GTT	AAT	ACT	AAC	ATG	GGT	TTA	AAG	ATC	AGG	CAA
	CTA	TTG	TGG	TTT	CAT	ATA	TCT	TGC	CTT	ACT	TTT
	GGA	AGA	GAG	ACT	GTA	CTT	GAA	TAT	TTG	GTC	TCT
40	TTC	GGA	GTG	TGG	ATT	CGC	ACT	CCT	CCA	GCC	TAT
	AGA	CCA	CCA	AAT	GCC	CCT	ATG	TTA	TCA	ACA	CTT
45	CCG	GAA	ACT	ACT	GTT	GTT	AGA	CGA	CGG	GAC	CGA
	GGC	AGG	TCC	CCT	AGA	AGA	AGA	ACT	CCC	TCG	CCT
	CGC	AGA	CGT	AGA	TCT	CAA	TCG	CCG	CGT	CGC	AGA
50	AGA	TCT	CAA	TCT	CGG	GAA	TCT	CAA	TGT	TAG	

Claims

1. A combination of
 - a) at least one polypeptide sequence mediating the antigenicity of one or more epitopes and
 - b) a carrier capable of presenting the epitope sequence(s) a), wherein the polypeptide sequence(s)

SEQ ID NO: 34
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 25 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

5'-CCT-GTA-GAA-CCA-AAT-ATT-CAA-GTA-(G)-T

5 SEQ ID NO: 32
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 89 bp
STRANDEDNESS: single
10 TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
15 IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

20
3'-GAT-CTC-CTA-GAC-ACC-GCC-TCA-GCT-CTG-T
25 AT-CGA-GAA-GCC-TTA-GAG-TCT-CCT-GAG-CAT-T
GC-TCA-CCT-CAC-CAT-ACG-GCA-CTC-AGG-CAA-G
-(G)-3'

35 SEQ ID NO: 33
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 25 bp
STRANDEDNESS: single
40 TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
45 IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

50
3'-TTG-GAT-CCT-CGA-ACC-TGT-GCC-TTG-(G)-3'

SEQ ID NO: 30
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 108 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

5'-GAT-CTT-TTA-AAG-GGA-TCC-TCT-GCT-GGG-G
 GG-AAT-GGA-TGA-CTC-TAG-CTA-CCT-GGG-TGG-G
 CA-ATA-ATT-TGG-AAG-ATC-CAG-CAT-CTA-GGG-A
 CC-TTG-TAG-TAA-ATC-TAG-AC-(A)-3'

SEQ ID NO: 31
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 106 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

5'-GAT-CTC-CGG-GAA-TTC-CTG-GGG-CAT-GGA-C
 AT-TGA-CGC-TTA-TAA-AGA-ATT-TGG-AGC-TAC-T
 GT-GGA-GTT-ACT-CTC-GTT-TTT-GCC-TTC-TGA-C
 TT-CTT-TCC-TTC-CGT-CAG-(G)-3'

SEQ ID NO: 28
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 106 bp
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV S1
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

5'-GAT-CTT-TAA-CAT-GGA-GAA-CAA-TCC-TCT-G
GG-ATT-CTT-TCC-GGA-TCA-CCA-GTT-GGA-TCC-A
GC-CTT-CAG-AGC-AAA-CAC-CGC-AAA-TCC-AGA-T
TG-GGA-CTT-CAA-TCC-CAG-(T)-3'

SEQ ID NO: 29
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 115 bp
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV S
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

5'-AAT-TCT-AGA-CTC-GAG-TCT-GAA-CAT-AGA-G
AA-CAT-CAC-ATC-AGG-ATT-CCT-AGG-ACC-CCT-T
CT-CGT-GTT-ACA-GGC-GGG-GTT-TTT-CTT-GTT-G
AC-AAG-AAT-CCT-CAC-AAT-ACC-GCA-GAG-(C)-3'

SEQ ID NO: 27
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 585 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV S adw/ayw
 IMMEDIATE EXPERIMENTAL
 SOURCE: HBV DNA

```

CTA GAC TCG TGG TGG ACT TCT CTC AAT TTT CTA GGG
GGA TCT CCC GTG TGT CTT GGC CAA AAT TCG CAG TCC
CCA ACC TCC AAT CAC TCA CCA ACC TCC TGT CCT CCA
ATT TGT CCT GGT TAT CGC TGG ATG TGT CTG CGG CGT
TTT ATC ATA TTC CTC TTC ATC CTG CTG CTA TGC CTC
ATC TTC TTA TTG GTT CTT CTG GAT TAT CAA GGT ATG
TTG CCC GTT TGT CCT CTA ATT CCA GGA TCA ACA ACA
ACC AGT ACG GGA CCA TGC AAA ACC TGC ACG ACT CCT
GCT CAA GGC AAC TCT ATG TTT CCC TCA TGT TGC TGT
ACA AAA CCT ACG GAT GGA AAT TGC ACC TGT ATT CCC
ATC CCA TCG TCC TGG GCT TTC GCA AAA TAC CTA TGG
GAG TGG GCC TCA GTC CGT TTC TCT TGG CTC AGT TTA
CTA GTG CCA TTT GTT CAG TGG TTC GTA GGG CTT TCC
CCC ACT GTT TGG CTT TCA GCT ATA TGG ATG ATG TGG
TAT TGG GGG CCA AGT CTG TAC AGC ATC GTG AGT CCC
TTT ATA CCG CTG TTA CCA ATT TTC TTT TGT CTC TGG
GTA TAC ATT

```

SEQ ID NO:	26
SEQ TYPE:	Nucleotide
SEQUENCE LENGTH:	678 bp
STRANDEDNESS:	single
TOPOLOGY:	linear
MOLECULE TYPE:	genomic DNA
ORIGINAL SOURCE:	HBV S adw/ayw
IMMEDIATE EXPERIMENTAL	
SOURCE:	HBV DNA

ATA	GAG	AAC	ATC	ACA	TCA	GGA	TTC	CTA	GGA	CCC	CTT	CTC
GTG	TTA	CAG	GCG	GGG	TTT	TTC	TTG	TTG	ACA	AGA	ATC	CTC
ACA	ATA	CCG	CAG	AGT	CTA	GAC	TCG	TGG	TGG	ACT	TCT	CTC
AAT	TTT	CTA	GGG	GGA	ACT	ACC	GTG	TGT	CTT	GGC	CAA	AAT
TCG	CAG	TCC	TCA	ACC	TCC	AAT	CAC	TCA	CCA	ACC	TCT	TGT
CCT	CCA	ACT	TGT	CCT	GGT	TAT	CGC	TGG	ATG	TGT	CTG	CGG
CGT	TTT	ATC	ATC	TTC	CTC	TTC	ATC	CTG	CTG	CTA	TGC	CTC
ATC	TTC	TTG	TTG	GTT	CTT	CTG	GAC	TAT	CAA	GGT	ATG	TTG
CCC	GTT	TGT	CCT	CTA	ATT	CCA	GGA	TCC	TCA	ACA	ACC	AGC
ACG	GGA	CCA	TGC	CGG	ACC	TGC	ATG	ACT	ACT	GCT	CAA	GGA
ACC	TCT	ATG	TAT	CCC	TCC	TGT	TGC	TGT	ACC	AAA	CCT	TCG
GAC	GGA	AAT	TGC	ACC	TGT	ATT	CCC	ATC	CCA	TCA	TCC	TGG
GCT	TTC	GGA	AAA	TTC	CTA	TGG	GAG	TGG	GCC	TCA	GCC	CGT
TTC	TCC	TGG	CTC	AGT	TTA	CTA	GTG	CCA	TTT	GTT	CAG	TGG
TTC	GTA	GGG	CTT	TCC	CCC	ACT	GTT	TGG	CTT	TCA	GTT	ATA
TGG	ATG	ATG	TGG	TAT	TGG	GGG	CCA	AGT	CTG	TAC	AGC	ATC
TTG	AGT	CCC	TTT	TTA	CCG	CTG	TTA	CCA	ATT	TTC	TTT	TGT
CTT	TGG	GTA	TAC	ATT								

SEQ ID NO: 25
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 558 bp
 5 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 10 ORIGINAL SOURCE: HBV core adw
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

15
 20 ATG GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT
 ACT GTG GAG TTA CTC TCG TTT TTG CCT TCT GAC
 TTC TTT CCT TCC GTA CGA GAT CTC CTA GAC ACC
 25 GCC TCA GCT CTG TAT CGA GAA GCC TTA GAG TCT
 CCT GAG CAT TGC TCA CCT CAC CAT ACT GCA CTC
 AGG CAA GCC ATT CTC TGC TGG GGG GAA TTG ATG
 30 ACT CTA GCT ACC TGG GTG GGT AAT AAT TTG CAA
 GAT CCA GCA TCC AGA GAT CTA GTA GTC AAT TAT
 GTT AAT ACT AAC ATG GGT TTA AAG ATC AGG CAA
 35 CTA TTG TGG TTT CAT ATA TCT TGC CTT ACT TTT
 GGA -AGA GAG ACT GTA CTT GAA TAT TTG GTC TCT
 TTC GGA GTG TGG ATT CGC ACT CCT CCA GCC TAT
 40 AGA CCA CCA AAT GCC CCT ATG TTA TCA ACA CTT
 CCG GAA ACT ACT GTT GTT AGA CGA CGG GAC CGA
 GGC AGG TCC CCT AGA AGA AGA ACT CCC TCG CCT
 45 CGC AGA CGT AGA TCT CAA TCG CCG CGT CGC AGA
 AGA TCT CAA TCT CGG GAA TCT CAA TGT TAG

50

55

SEQ ID NO: 23
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 60 bp
5 STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
10 ORIGINAL SOURCE: HBV S2 ay
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

20 CAG TGG AAT TCC AGA ACC TTC CAC CAA ACT CTG
CAA GAT CCC AGA GTG AGA GGC CTG TAT - X

25
30 SEQ ID NO: 24
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 60 bp
STRANDEDNESS: single
35 TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV S2 ay
IMMEDIATE EXPERIMENTAL
40 SOURCE: chemically synthesized

45 GAT CCC AGA GTG AGA GGC CTG TAT TTC CCT GCT
GGT GGC TCC AGT TCA GGA ACA GTA AAC - X

SEQ ID NO: 22
SEQ TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 108 bp
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV S1 ad
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

CCA CAC GGC GGT ATT TTG GGG TGG AGC CCT CAG GCT
CAG GGC ATA TTG ACC ACA GTG TCA ACA ATT CCT CCT
CCT GCC TCC ACC AAT CCG CAG TCA GGA AGG CAG CCT

Pro His Gly Gly Ile Leu Gly Trp Ser Pro Gln
Ala Gln Gly Ile Leu Thr Thr Val Ser Thr Ile
Pro Pro Pro Ala Ser Thr Asn Arg Gln Ser Gly
Arg Gln Pro

SEQ ID NO: 21
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 63 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV S1 ad
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

CCT GCC TCC ACC AAT CGG CAG TCA GGA AGG CAG CCT

ACT CCC ATC TCT CCA CCT CTA AGA GAC - X

SEQ ID NO: 20
SEQ TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 108 bp
5 STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
10 ORIGINAL SOURCE: HBV S1 ay
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

15
CCG CAC GGA GGC CTT TTG GGG TGG AGC CCT CAG GCT

20 CAG GGC ATA CTA CAA ACT TTG CCA GCA AAT CCG CCT

CCT GCC TCC ACC AAT CGC CAG TCA GGA AGG CAG CCT

25
Pro His Gly Gly Leu Leu Gly Trp Ser Pro Gln

30 Ala Gln Gly Ile Leu Glu Thr Leu Pro Ala Asn

Pro Pro Pro Ala Ser Thr Asn Arg Gln Ser Gly

35 Arg Gln Pro

SEQ ID NO: 19
SEQ TYPE: Nucleotide with corresponding protein
SEQUENCE LENGTH: 63 bp
5 STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
10 ORIGINAL SOURCE: HBV S1 ay
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

20 GAT CCA GCC TTC AGA GCA AAC ACC GCA AAT CCA GAT
TGG GAC TTC AAT CCC AAC AAG GAC ACC

25
Asp Pro Ala Phe Arg Ala Asn Thr Ala Asn Pro Asp
30 Trp Asp Phe Asn Pro Asn Lys Asp Thr

35

40

45

50

55

SEQ ID NO: 18
 SEQ TYPE: Nucleotide with corresponding protein
 SEQUENCE LENGTH: 63 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV S1 ay
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

CCT GCC TCC ACC AAT CGC CAG TCA GGA AGG CAG CCT

ACC CCG CTG TCT CCA CCT TTG AGA AAC

Pro Ala Ser Thr Asn Arg Gln Ser Gly Arg Gln Pro

Thr Pro Ile Ser Pro Pro Leu Arg Asn

SEQ ID NO: 17
SEQ TYPE: Nucleotide with corresponding protein
5 SEQUENCE LENGTH: 60 bp
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
10 ORIGINAL SOURCE: HBV S1 ay
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

20 AAT CCT CTG GGA TTC TTT CCC GAT CAC CAG TTG GAT
CCA GCC TTC AGA GCA AAC ACC GCA

25
30 Asn Pro Leu Gly Phe Phe Pro Asp His Gln Leu Asp
35 Pro Ala Phe Arg Ala Asn Thr Ala

SEQ ID NO: 16
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 390 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

	TCC	AAC	CTG	TGC	CTT	GGG	TGG	CTT	TGG	GGC
20	ATG	GAC	ATT	GAC	CCT	TAT	AAA	GAA	TTT	GGA
	ACT	GTG	GAG	TTA	CTC	TCG	TTT	TTG	CCT	TCT
	TTC	TTT	CCT	TCC	GTA	CGA	GAT	CTC	CTA	GAC
25	GCC	TCA	GCT	CTG	TAT	CGA	GAA	GCC	TTA	GAG
	CCT	GAG	CAT	TGC	TCA	CCT	CAC	CAT	ACT	GCA
30	AGG	CAA	GCC	ATT	CTC	TGC	TGG	GGG	GAA	TTG
	ACT	CTA	GCT	ACC	TGG	GTG	GGT	AAT	AAT	TTG
	GAT	CCA	GCA	TCC	AGA	GAT	CTA	GTA	GTC	AAT
35	GTT	AAT	ACT	AAC	ATG	GGT	TTA	AAG	ATC	AGG
	CTA	TTG	TGG	TTT	CAT	ATA	TCT	TGC	CTT	ACT
40	GGA	AGA	GAG	ACT	GTA	CTT	GAA	TAT	TTG	GTC

SEQ ID NO: 15
 SEQ TYPE: Nucleotide
 5 SEQUENCE LENGTH: 99 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 10 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 15 SOURCE: chemically synthesized

20 TAT GTT AAT ACT AAC ATG GGT TTA AAG ATC AGG
 CAA CTA TTG TGG TTT CAT ATA TCT TGC CTT ACT
 TTT GGA AGA GAG ACT GTA CTT GAA TAT TTG GTC

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SEQ ID NO: 14
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 822 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV S2 ayw/adw
 IMMEDIATE EXPERIMENTAL
 SOURCE: HBV DNA

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    ATG CAG TGG AAT TCC AGA ACC TTC CAC CAA ACT CTG
    CAA GAT CCC AGA GTG AGA GGC CTG TAT TTC CCT GCT
    GGT GGC TCC AGT TCA GGA ACA GTA AAC CCT GTT CTG
    ACT ACT GCC TCT CCC TTA TCG TCA ATC TTC TCG AGG
    ATA GAG AAC ATC ACA TCA GGA TTC CTA GGA CCC CTT
    CTC GTG TTA CAG GCG GGG TTT TTC TTG TTG ACA AGA
    ATC CTC ACA ATA CCG CAG AGT CTA GAC TCG TGG TGG
    ACT TCT CTC AAT TTT CTA GGG GGA ACT ACC GTG TGT
    CTT GGC CAA AAT TCG CAG TCC TCA ACC TCC AAT CAC
    TCA CCA ACC TCT TGT CCT CCA ACT TGT CCT GGT TAT
    CGC TGG ATG TGT CTG CGG CGT TTT ATC ATC TTC CTC
    TTC ATC CTG CTG CTA TGC CTC ATC TTC TTG TTG GTT
    CTT CTG GAC TAT CAA GGT ATG TTG CCC GTT TGT CCT
    CTA ATT CCA GGA TCC TCA ACA ACC AGC ACG GGA CCA
    TGC CGG ACC TGC ATG ACT ACT GCT CAA GGA ACC TCT
    ATG TAT CCC TCC TGT TGC TGT ACC AAA CCT TCG GAC
    GGA AAT TGC ACC TGT ATT CCC ATC CCA TCA TCC TGG
    GCT TTC GGA AAA TTC CTA TGG GAG TGG GCC TCA GCC
    CGT TTC TCC TGG CTC AGT TTA CTA GTG CCA TTT GTT
    CAG TGG TTC GTA GGG CTT TCC CCC ACT GTT TGG CTT
    TCA GTT ATA TGG ATG ATG TGG TAT TGG GGG CCA AGT
    CTG TAC AGC ATC TTG AGT CCC TTT TTA CCG CTG TTA
    CCA ATT TTC TTT TGT CTT TGG GTA TAC ATT
  
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EP 0 491 077 A1

SEQ ID NO: 12
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 123 bp
STRANDEDNESS: single
5 TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
10 IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

15 ACC TGG GTG GGT AAT AAT TTG CAA GAT CCA GCA
TCC AGA GAT CTA GTA GTC AAT TAT GTT AAT ACT
AAC ATG GGT TTA AAG ATC AGG CAA CTA TTG TGG
20 TTT CAT ATA TCT TGC CTT ACT TTT

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30 SEQ ID NO: 13
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 138 bp
35 STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
40 IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

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50 GCA TCC AGA GAT CTA GTA GTC AAT TAT GTT AAT
ACT AAC ATG GGT TTA AAG ATC AGG CAA CTA TTG
TGG TTT CAT ATA TCT TGC CTT ACT TTT GGA AGA
GAG ACT GTA CTT GAA TAT TTG GTC TCT TTC GGA
55 GTG TGG

SEQ ID NO: 11
SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 291 bp
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

TCC	AAC	CTG	TGC	CTT	GGG	TGG	CTT	TGG	GGC	
ATG	GAC	ATT	GAC	CCT	TAT	AAA	GAA	TTT	GGA	GCT
ACT	GTG	GAG	TTA	CTC	TCG	TTT	TTG	CCT	TCT	GAC
TTC	TTT	CCT	TCC	GTC	AGG	GAT	CTC	CTA	GAC	ACC
GCC	TCA	GCT	CTG	TAT	CGA	GAA	GCC	TTA	GAG	TCT
CCT	GAG	CTA	TGC	TCA	CCT	CAC	CAT	ACT	GCA	CTC
AGG	CAA	GGT	ATC	CTC	TGC	TGG	GGG	GAA	TGG	ATG
ACT	CTA	GCT	ACC	TGG	GTG	GGC	AAT	AAT	TTG	GAA
GAT	CCA	GCA	TCT	AGG	GAC	CTT	GTA	GTA	AAT	

SEQ ID NO: 10
5 SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 261 bp
STRANDEDNESS: single
10 TOPOLOGY: linear
MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
15 IMMEDIATE EXPERIMENTAL
SOURCE: chemically synthesized

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ATG GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT

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ACT GTG GAG TTA CTC TCG TTT TTG CCT TCT GAC

TTC TTT CCT TCC GTC AGG GAT CTC CTA GAC ACC

30

GCC TCA GCT CTG TAT CGA GAA GCC TTA GAG TCT

CCT GAG CTA TGC TCA CCT CAC CAT ACT GCA CTC

35

AGG CAA GGT ATC CTC TGC TGG GGG GAA TGG ATG

ACT CTA GCT ACC TGG GTG GGC AAT AAT TTG GAA

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GAT CCA GCA TCT AGG GAC CTT GTA GTA AAT

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SEQ ID NO: 8
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 114 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

TCC AAC CTG TGC CTT GGG TGG CTT TGG GGC ATG
 GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT ACT
 GTG GAG TTA CTC TCG TTT TTG CCT TCT GAC TTC
 TTT CCT TCC GTC AGG

SEQ ID NO: 9
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 90 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: chemically synthesized

GAT CTC CTA GAC ACC GCC TCA GCT CTG TAT CGA
 GAA GCC TTA GAG TCT CCT GAG CTA TGC TCA CCT
 CAC CAT ACT GCA CTC AGG CAA GGT

SEQ ID NO: 6
5 SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 87 bp
STRANDEDNESS: single
TOPOLOGY: linear
10 MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
IMMEDIATE EXPERIMENTAL
15 SOURCE: chemically synthesized

20 ATC CTC TGC TGG GGG GAA TGG ATG ACT CTA GCT
ACC TGG GTG GGC AAT AAT TTG GAA GAT CCA GCA
25 TCT AGG GAC CTT GTA GTA AAT

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SEQ ID NO: 7
35 SEQ TYPE: Nucleotide
SEQUENCE LENGTH: 81 bp
STRANDEDNESS: single
TOPOLOGY: linear
40 MOLECULE TYPE: genomic DNA
ORIGINAL SOURCE: HBV core
IMMEDIATE EXPERIMENTAL
45 SOURCE: chemically synthesized

50 GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT ACT
GTG GAG TTA CTC TCG TTT TTG CCT TCT GAC TTC
55 TTT CCT TCC GTC AGG

SEQ ID NO: 5
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 534 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

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20      TCC AAC CTG TGC CTT GGG TGG CTT TGG GGC
      ATG GAC ATT GAC CCT TAT AAA GAA TTT GGA GCT
      ACT GTG GAG TTA CTC TCG TTT TTG CCT TCT GAC
25      TTC TTT CCT TCC GTA CGA GAT CTC CTA GAC ACC
      GCC TCA GCT CTG TAT CGA GAA GCC TTA GAG TCT
      CCT GAG CAT TGC TCA CCT CAC CAT ACT GCA CTC
30      AGG CAA GCC ATT CTC TGC TGG GGG GAA TTG ATG
      ACT CTA GCT ACC TGG GTG GGT AAT AAT TTG CAA
35      GAT CCA GCA TCC AGA GAT CTA GTA GTC AAT TAT
      GTT - AAT ACT AAC ATG GGT TTA AAG ATC AGG CAA
      CTA TTG TGG TTT CAT ATA TCT TGC CTT ACT TTT
40      GGA AGA GAG ACT GTA CTT GAA TAT TTG GTC TCT
      TTC GGA GTG TGG ATT CGC ACT CCT CCA GCC TAT
      AGA CCA CCA AAT GCC CCT ATG TTA TCA ACA CTT
45      CCG GAA ACT ACT GTT GTT AGA CGA CGG GAC CGA
      GGC AGG TCC CCT AGA AGA AGA ACT CCC TCG CCT
50      CGC AGA CGT
  
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SEQ ID NO: 4
 SEQ TYPE: Nucleotide
 SEQUENCE LENGTH: 534 bp
 STRANDEDNESS: single
 TOPOLOGY: linear
 MOLECULE TYPE: genomic DNA
 ORIGINAL SOURCE: HBV core
 IMMEDIATE EXPERIMENTAL
 SOURCE: PCR-amplification

20	TCC	AAC	CTG	TGC	CTT	GGG	TGG	CTT	TGG	GGC
	ATG	GAC	ATT	GAC	CCT	TAT	AAA	GAA	TTT	GGA
	ACT	GTG	GAG	TTA	CTC	TCG	TTT	TTG	CCT	TCT
25	TTC	TTT	CCT	TCC	GTA	CGA	GAT	CTC	CTA	GAC
	GCC	TCA	GCT	CTG	TAT	CGA	GAA	GCC	TTA	GAG
	CCT	GAG	CAT	TGC	TCA	CCT	CAC	CAT	ACT	GCA
30	AGG	CAA	GCC	ATT	CTC	TGC	TGG	GGG	GAA	TTG
	ACT	CTA	GCT	ACC	TGG	GTG	GGT	AAT	AAT	TTG
	GAT	CCA	GCA	TCC	AGA	GAT	CTA	GTA	GTC	AAT
35	GTT	AAT	ACT	AAC	ATG	GGT	TTA	AAG	ATC	AGG
	CTA	TTG	TGG	TTT	CAT	ATA	TCT	TGC	CTT	ACT
40	GGA	AGA	GAG	ACT	GTA	CTT	GAA	TAT	TTG	GTC
	TTC	GGA	GTG	TGG	ATT	CGC	ACT	CCT	CCA	GCC
	AGA	CCA	CCA	AAT	GCC	CCT	ATG	TTA	TCA	ACA
45	CCG	GAA	ACT	ACT	GTT	GTT	AGA	CGA	CGG	GAC
	GGC	AGG	TCC	CCT	AGA	AGA	AGA	ACT	CCC	TCG
50	CGC	AGA	CGT							